AN INVESTIGATION OF ARSENIC IN BIOLOGICAL SAMPLES FROM UNEXPOSED VOLUNTEERS IN THE UK

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The contribution of my Mum Batoul and my Dad Ibrahim cannot be underestimated, without their love and prayer I would never have made it this far.

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Abstract

This thesis describes studies on the analysis of arsenic (As) in human biological samples, mainly urine but also hair and fingernails using inductively coupled plasma mass spectrometry (ICP-MS) and graphite furnace atomic absorption spectrometry (GF-AAS). The relationship between ethnicity and arsenic metabolism was investigated for the first time for a population in the United Kingdom. This investigation has been carried out through comparative analysis of arsenic in human urine, hair and fingernails in volunteers from three different ethnic groups (Whites, Asians and Somali Black-Africans) who are only exposed to background levels of arsenic. Results obtained with 63 volunteers showed ethnic differences in urinary arsenic excretion as well as differences in arsenic levels in fingernail samples. The averages of total arsenic levels for the Somali Black-Africans (urine 7.2 µg/g creatinine; fingernails 723 µg/kg) are significantly ($P< 0.05$) different from both the Asians (urine 20.6 µg/g creatinine; fingernails 153.9 µg/kg) and Whites (urine 24.5 µg/g creatinine; fingernails 177.0 µg/kg). The Somali group also shows a higher percentage (50%) of dimethylarsinate (DMA) and a lower percentage (48%) of arsenobetaine (AB), compared to Asians (16% DMA and 83% AB) and Whites (22% DMA and 77% AB).

The effect of fasting on urinary arsenic species distribution was also investigated by monitoring urine samples from 29 Ramadan fasting volunteers, with each volunteer providing a sample at the beginning (RF1) and at the end (RF2) of an approximately 12 hours fast. The results obtained showed the frequency of MA

detection for RF2 was 12 and 2-fold higher than for the non-fasting and RF1 groups, respectively. This suggests fasting may alter the pattern of arsenic metabolism and excretion. However, there was no significant difference ($P > 0.05$) in the average of total level of arsenic for RF1 (18.3 µg/g creatinine) and RF2 (17.7 µg/g creatinine).

A relationship between excretion of arsenic and selenium in individuals exposed to background levels of arsenic and selenium was investigated through analysis of urine samples from 93 volunteers from Leicester, UK. A positive correlation between arsenic and selenium was found and the As:Se ratio was 0.7 ± 0.4. The intra-individual variation of As:Se ratio does not alter significantly over time, as determined by monitoring urine samples from a volunteer over a period of one year. Furthermore, within a single day, with urine samples collected at the beginning and after a 12-hour fast, the As:Se ratio was found to be similar (0.7 ± 0.5). These findings suggest a close relationship between these two metalloids, the biological significance of which needs to be explored in the future.

Contents

Publications

Published Papers:

1- **Brima, E. I.**, Haris, P.I., Jenkins, R.O., Polya, D.A., Gault, A.G., Harrington, C.F., 2006. Understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicol. Appl. Pharmacol.*, **216**, 122 -130.

2- **Brima, E. I.,** Jenkins, R.O., Haris, P.I., 2006. Understanding arsenic metabolism through spectroscopic determination of arsenic in human urine. *Spectrosc- Int. J.,* **20**, 125 – 151.

3- **Brima, E. I.**, Jenkins, R.O., Gault, A.G., Polya, D.A., Haris, P.I., 2007. The effect of fasting on the pattern of urinary arsenic excretion.. *J. Environ. Monit.,* **9**, 98 – 104.

Submitted Papers:

4- **Brima, E. I.,** Harrington, C.F., Jenkins, R.O., Gault, A.G., Polya, D.A., Pearson, G. F., Greenway, G.M., Haris, P.I., 2007. Establishing a baseline value for urinary arsenic: selenium ratio in unexposed populations in the United Kingdom. *Spectrosc- Int. J.,* (submitted).

Conferences:

1- 16th International Mass Spectrometry Conference, Edinburgh,UK. 31st August – 5th September 2003**:** ***Poster:***

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Quantification of arsenobetaine in urine by using solid phase extraction with liquid chromatography mass spectrometry.

2- 27th Annual Meeting 5th – 8th September 2004, Derby, UK. British Mass Spectrometry Society**:** ***Poster:***

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Urinary arsenic species among the population of Leicester, UK.

3- Life at the research Frontier, The British Association, 25th October 2004, Leicester,UK: ***Oral presentation:***

Brima, E. I. Jenkins R.O. Haris P.I.

Arsenic content in human urine as a function of diet and ethnicity.

4- 7th ICEBAMO, International Conference on Environmental and Biological Aspects of Main-Group Organometallics. Heraklion, Crete, Greece. 10th - 12th October 2006, **Poster:**

Harrington, C.F., **Brima, E. I.**, Haris P.I., Jenkins R.O.

The degradation of arsenobetaine by human fecal microorganisms

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List of Abbreviations

AAS	Atomic absorption spectrometry
AB	Arsenobetaine
AC	Arsenocholine
AFS	Atomic fluorescence spectrometry
As (III)	Arsenite
As (V)	Arsenate
DMA	Dimethylarsinate
DMAG	dimethylarsinic glutathione
DMA (III)	Dimethylarsinite
DMAA	Dimethylarsinoyl acetic acid
DMAE	Dimethylarsinoylethanol
DMSe	Dimethylselenide
GF-AAS	Graphite furnace atomic absorption spectrometry
$[(GS)_2AsSe]^-$	Seleno-bis(*S*-glutathionyl) arsinium ion
GSH	Glutathione
HG	Hydride generation
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
m/z	Mass to charge ratio
MA	Methylarsonate
MADG	Monomethylarsonic diglutathione
MA (III)	Methylarsonite

MS	Mass Spectrometry
MSe	Monomethylated selenium
SAM	*S*-adenosyl methionine
Se (IV)	Selenite
TETRA	Tetramethylarsonium ion
TMAO	Trimethylarsine oxide
TMSe	Trimethylarsenium

Chapter 1

Introduction

Millions of people worldwide suffer from chronic exposure to inorganic arsenic (As) through their drinking water (Samanta et al., 1999). In Bangladesh and India alone over 100 million people are affected (Tokunaga et al., 2002). Arsenic in the environment can exist as organic and inorganic compounds. Exposure to inorganic arsenic is associated with various health effects such as cancer of skin, lung, kidney, liver and bladder; skin manifestations such as melanosis and keratosis have also been reported (Samanta et al., 2000). Urinary excretion is the major pathway for the elimination of arsenic compounds from the human body (Le at al., 1994). Therefore, urinary arsenic can be used as a biomarker of exposure to arsenic in humans, although arsenic in hair and nail also can also be used. Arsenic in urine reflects only recent exposure while arsenic in hair and nail represent chronic exposure (Mandal et al., 2004). This thesis focuses mainly on the analysis of arsenic in human urine.

1.1 Arsenic Chemistry

Arsenic (As) has the atomic number and atomic mass of 33 and 74.92 respectively, as shown in Table 1.1 (Lenntech, 2007). Arsenic is a metalloid widely distributed in the environment. Arsenic occurs with a valence state of +3 and +5, with rare occurrences of -3 and 0. For this reason, + 3 (arsenite (As (III)) and +5 (arsenate

(As (V)) are the main species that dominate studies of arsenic in chemistry (Kirk-Othmer, 1992).

Table 1.1 Chemical properties of arsenic (compiled from Lenntech, 2007).

Atomic number	33
Atomic mass	74.9216 g.mol $^{-1}$
Electronic shell	[Ar] $3d^{10}$ $4s^2$ $4p^3$
Melting point	814 °C (36 atm.)
Boiling point	615 °C (sublimation)
Density	5.7 g.cm^{-3} at 14°C
Standard potential	- 0.3 V (As^{3+}/ As)
Electronegativity	2.0
Ionic radius	0.222 nm (-2) 0,047 nm (+5) 0,058 (+3)
Energy of first ionisation	947 kJ.mol $^{-1}$
Energy of second ionisation	1798 kJ.mol $^{-1}$
Energy of first ionisation	947 kJ.mol $^{-1}$

Inorganic compounds of arsenic can occur as halides, hydride (arsine), oxides, acids, and sulphides (Kirk-Othmer, 1992). Different conditions can affect arsenic's valence state as well as its chemical forms such as pH, oxidation-reduction potential, microbial activity, and the presence of different ions, e.g. sulphur, iron, and calcium (Vance, 2007). In natural waters, arsenic occurs predominantly as As (III) and As (V) (Smedley and Kinniburgh, 2002). In oxidising environments the pentavalent forms H_3AsO_4, $H_2AsO_4^-$(aq), $HAsO_4^{2-}$(aq) and AsO_4^{3-}(aq) are predominant, whilst trivalent forms such as H_3AsO_3, $H_2AsO_3^-$(aq), $HAsO_3^{2-}$(aq) and AsO_3^{3-}(aq), are typically predominant species in reduced conditions (Smedley and Kinniburgh, 2002;

Bhattacharya et al., 2003). In these reduced conditions organic arsenic such as methylarsonate (MA) and dimethylarsinate (DMA) were also reported in groundwaters (Gault et al., 2003; Shraim et al., 2002). The degree of protonation of As (III) and As (V) is pH dependent. At low pK_a H_3AsO_3 occurs as neutrally charged species, whereas H_3AsO_4 occurs as the oxyanion species (Gault et al., 2005a). Redox potential (Eh) and pH are important factors for arsenic species (Smedley and Kinniburgh, 2002). An Eh-pH diagram for arsenic at 25^0C is shown in Fig. 1.1 (Ferguson and Gavis, 1972). The diagram results were obtained at one atmosphere with a total arsenic level of 10^{-5} mol/l and sulphur level of 10^{-3} mol/l (Ferguson and Gavis, 1972). At pH > 10 both As (III) and As (V) are present as anionic species, because the pK_{a1} values for both species (Table 1.3) are less than pH 10 (Yalcin and Le, 1998). As shown in Fig. 1.1, reduced and acidic conditions favour precipitation of As_2S_3 and AsS (Smedley and Kinniburgh, 2002; Cullen and Reimer 1989). Therefore, high levels of arsenic in water is not to be expected where there is a high concentration of free sulphide (Moore, 1988).

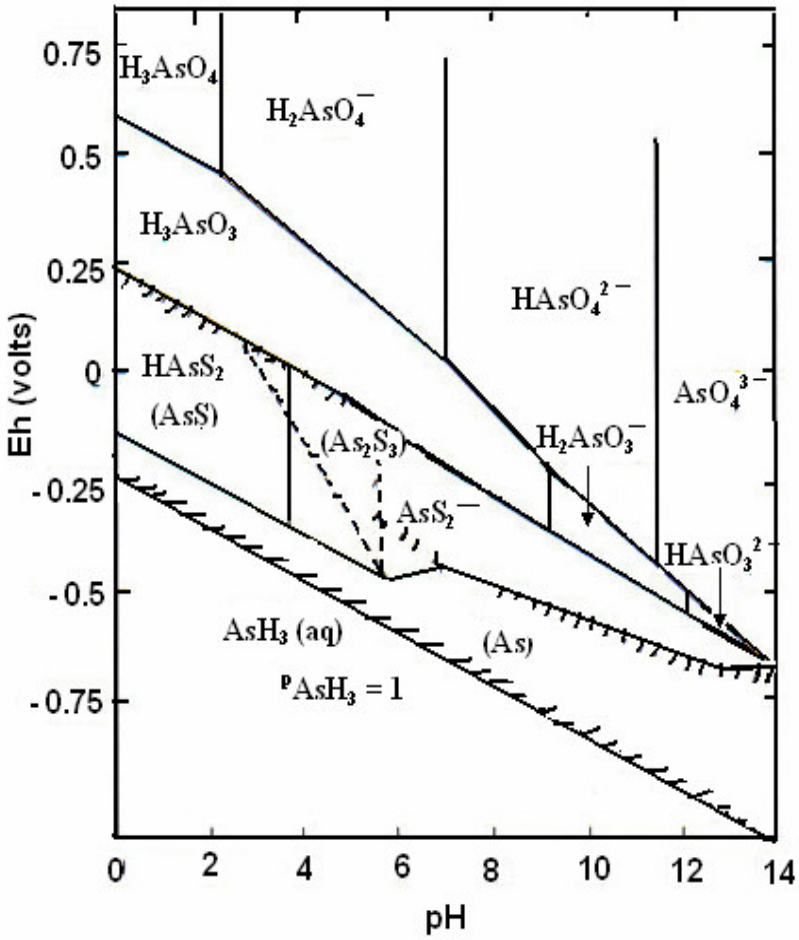


Figure 1.1 The Eh-pH diagram for arsenic (adapted from Ferguson and Gavis,1972)

Fig.1.2 shows the position of arsenic and selenium (Se) in the periodic table. These two elements appear close to each other in the table and it has been suggested that they interact with each other in humans (Levander, 1977). Section 1.4.1.6 discusses the effect of selenium on arsenic metabolism in humans.

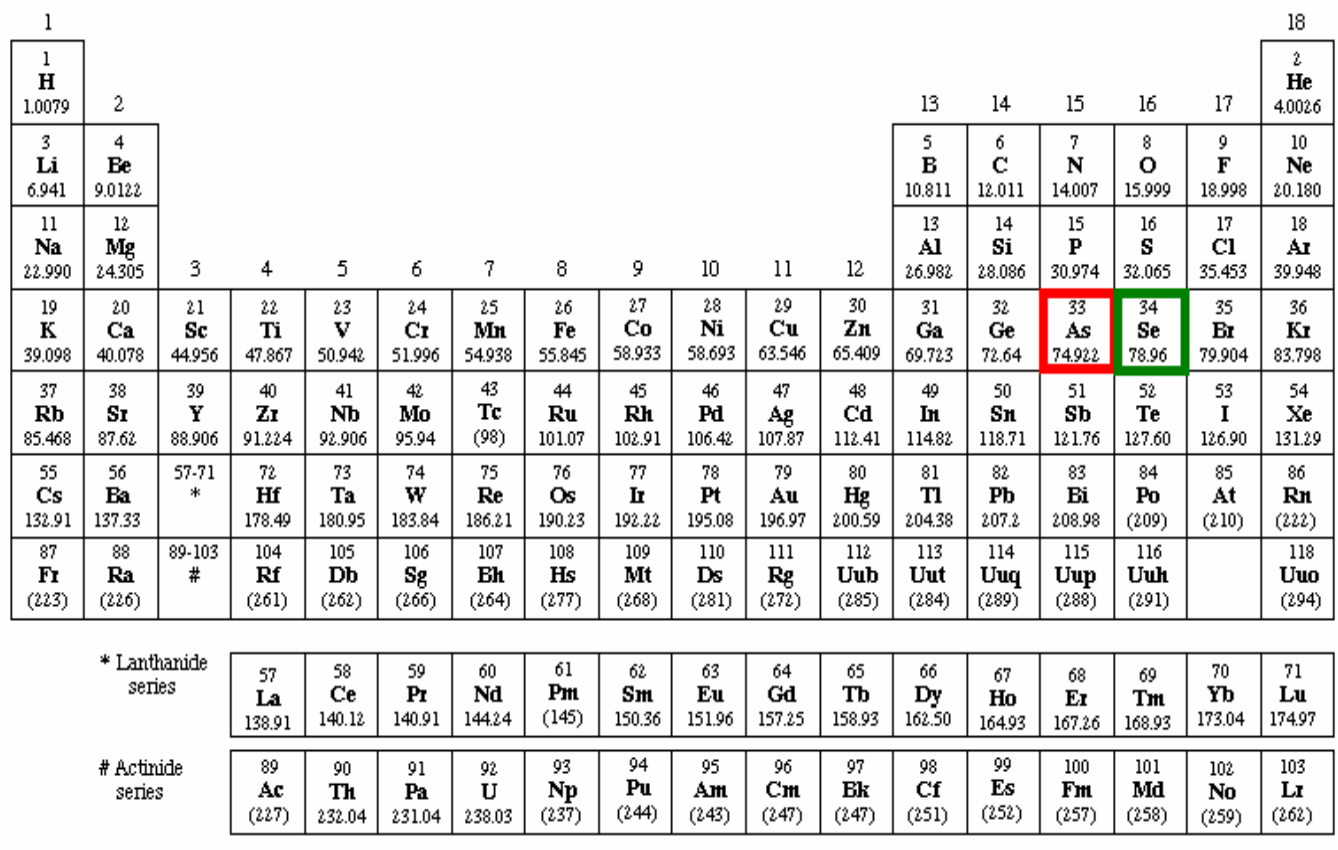


Figure 1.2 The Periodic Table with As and Se highlighted in red and green boxes, respectively

1.2 Arsenic occurrence

Arsenic can originate from both natural and anthropogenic sources; these are discussed separately below. Natural sources include wind-blown dust, volcanoes and seafood (Lima et al., 2002; Yamaushi et al., 1992), while examples of anthropogenic sources include insecticides (lead arsenate), herbicides (cacodylic acid), cotton desiccants (arsenic acid), wood preservatives (copper/chromium arsenate) and growth promoters for swine and poultry (substituted phenylarsonic acid) (Vahter, 1994).

1.2.1 Natural occurrence of arsenic

Arsenic occurs naturally in earth's crust, animals, soil, water, air, rocks and plants. Weathering of rocks results in arsenic release in the environment as a dust or in groundwater (Mandal and Suzuki, 2002; Smedley and Kinniburgh, 2002).

1.2.1.1 Arsenic in the earth's crust

The concentration of arsenic in the earth's crust is between 2 to 5 mg/kg, and it ranks 20th of all elements. The natural arsenic mineral consists of 60% arsenates, 20% sulphides and sulfosalts and 20% arsenides, arsenites, oxides and elemental arsenic (Smedley and Kinniburgh, 2002; Meerdink, 2001). The main mineral containing arsenic is sulphide, and the most abundant arsenic sulphide ores are arsenopyrite (FeAsS), realgar (As_4S_4) and orpiment (As_2S_3) (Smedley and Kinniburgh, 2002).

1.2.1.2 Arsenic in soil

Arsenic bound to organic materials in the soil mainly occurs as inorganic arsenic. As(V) is a predominant species under oxidizing conditions, while As(III) is the main species under reduced conditions (Bhumbla and Keefer, 1994). The concentration of arsenic in uncontaminated soils is from 0.1 to 40 mg/kg (Mandal and Suzuki, 2002; Pendias and Pendias, 1992). In contaminated soils, the range was found to be between 10 to 2470 mg/kg (Pendias and Pendias, 1992; Mandal and Suzuki, 2002).

1.2.1.3 Arsenic in water

Generally, the average arsenic concentration in seawater, river water and groundwater is 1.5, 0.1-0.8, and <10 µg/l (the background of arsenic concentration in most countries), respectively. However, a large range of arsenic concentration (< 0.5 – 5000 µg/l) has been reported in groundwater, which occurs under various natural conditions (Smedley and Kinniburgh, 2002).

Groundwater arsenic contamination has already been reported in 20 countries around the world (Fig. 1.3). The most affected countries, in order of severity, are Bangladesh, West Bengal (India), Inner Mongolia (China) and Taiwan (Chakraborti et al., 2002). Millions of people in arsenic-contaminated groundwater areas drink water with arsenic concentration of ≥ 50 µg/l, while the recommended guideline by the World Health Organization is 10 µg/l (Chakraborti et al., 2002; Smedley and Kinniburgh, 2002).

The mechanism blamed for releasing arsenic in groundwater is arsenic mobility, caused by weathering reaction and biological activity (Smedley and Kinniburgh, 2002). Breakdown of sulphide rocks containing arsenic convert arsenic to As (III), which enters the groundwater (Mandal et al., 2002). It is considered that a reductive dissolution of arsenic-bearing ferric hydroxides is driven by microbial degradation. Recently, Islam et al. (2004) showed that arsenic release took place after Fe(III) reduction, and that anaerobic metal-reducing bacteria can play a key role in the mobilisation of arsenic in sediments from a contaminated aquifer in West Bengal. In a study of a shallow aquifer in West Bengal, the microbial community at 24 and 45m depths was identified as the *Acinetobacter* species; in fact, this species comprises most of the 24m sedimentary bacterial population (Gault et al., 2005b).

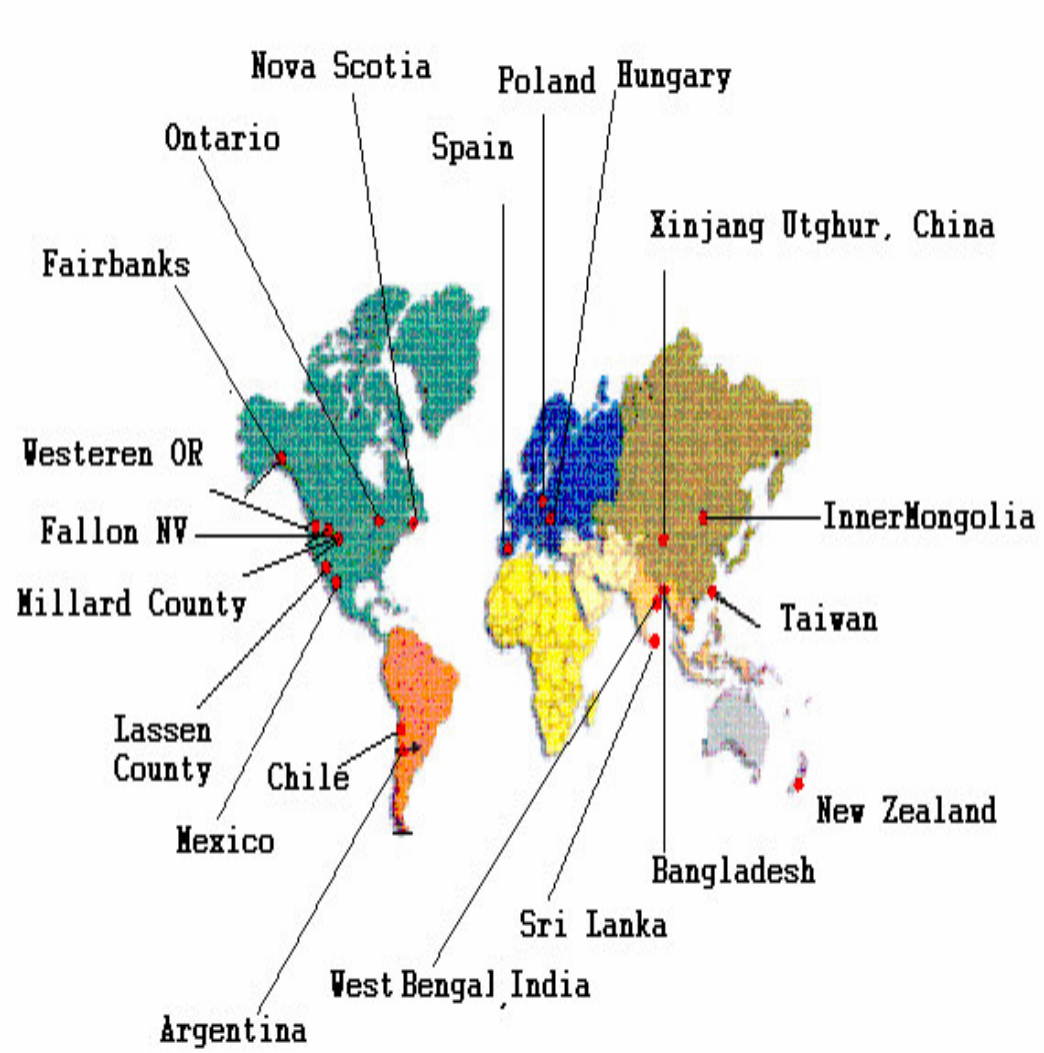


Figure 1. 3 Documented groundwater arsenic-contamination around the world, adapted from Chakraborti et al., (2002).

1.2.1.4 Arsenic in food

Most foods contain a low concentration of arsenic. Usually arsenic in food occurs in organic and inorganic forms, with organic forms dominating in fish and seafood (WHO, 1981). Arsenic in food is generally concentrated at < 1mg/kg wet weight, with the exception of some seafood. Marine fish contains arsenic on average < 5mg/kg. Seaweed from Japan has been reported to contain an average concentration of arsenic in the range of 19 -172 mg/kg dry weight. The use of arsenic containing feed additives for poultry and swine lead to accumulation of arsenic in these animals (WHO, 1981). Recently, organic arsenic was reported in rice and chicken (Williams et al., 2005; Polatajko et al., 2004). It has been reported in a review by Mandal et al. (2002) that fish, fruits and vegetables contain <10% inorganic arsenic, the remainder being mainly organic arsenic, while arsenic in some foods such as milk, beef, pork, poultry, dairy products and cereals is mainly inorganic, at an average of 65-75%.

1.2.1.5 Arsenic in Plants

In general, the concentration of arsenic in plants varies between <0.01 to 5 mg/g (dry weight basis) (Mandal and Suzuki, 2002). Different plant species can take up arsenic and then translocate it within various parts of the plant. The highest arsenic concentrations are always found to be in roots and old leaves (Pendias and Pendias, 1992). The concentration of arsenic varies from 0.009 to 1.5 mg/kg in plants grown in uncontaminated soils, the highest arsenic concentrations being recorded in leafy

vegetable and the lowest in fruits (Pendias and Pendias, 1992). The arsenic concentration in different vegetables from the UK and EU - purchased in Leicester City market - was reported to be in the range < 5 – 87 µg/kg (Al-Rmalli, 2004).

1.2.1.6 Arsenic in the human body

In general, 98.5% of the human body is made up of six elements. These are, in increasing order of abundance within the body, oxygen, carbon, hydrogen, nitrogen, calcium and phosphorus. Other elements such as potassium, sulphur, sodium and magnesium represent 0.8% while copper, zinc, selenium, molybdenum, fluorine, chlorine, iodine, manganese, cobalt and iron represent 0.7%. Arsenic and a range of other elements (lithium, strontium, aluminium, silicon, lead, vanadium, and bromine) only occur in trace amounts in the human body (Harper et al., 1977).

In humans, arsenic can accumulate particularly in ectodermic tissues such as hair and nails. The human body contains between 3 to 4 mg arsenic (Table 1.2), a figure which tends to increase with age (WHO, 2001). Most tissues in human body contain less than 0.3-47 µg/g (dry weight), with the exception of teeth, hair and nails (WHO, 2001). Anionic and soluble arsenic species are more highly absorbed by the human body than are insoluble species (Mandal and Suzuki, 2002).

Unexposed individuals in Europe have approximately 10-20 µg/l of arsenic in their urine (Ritsma et al., 1998; Stoeppler et al., 1994; Cornelis et al., 1995). The normal levels of arsenic in different biological samples (urine, hair and nail) have been reported as five to 40 µg per 1.5 l per day in urine (Farmer and Johnson, 1990), 80 to 250 µg/kg in hair (Arnold et al., 1990) and 430 to 1080 µg/kg in nail (Ioanid et al.,

1961). In the human blood, an arsenic concentration of 5.1 ± 6.9 µg/l was reported as an average level in normal individuals (Foa et al., 1984).

Table 1.2 Background levels of total arsenic in human body, biological samples and the natural environment

Material	**Arsenic concentration**	**Reference**
Whole human body	3 - 4 mg	WHO, 2001
Whole human body	0.3-147µg/g(dry weight), with the exception of teeth, hair and nails	WHO, 2001
Human urine	10-20 µg/l	Ritsema et al., 1998
Human urine	5 to 40 µg per 1.5 l (per day)	Farmer and Johnson, 1990
Human hair	80 to 250 µg/kg	Arnold et al., 1990
Human nail	430 to 1080 µg /kg	Ioanid et al., 1961
Human blood	5.1 ± 6.9 µg/l	Foa et al., 1984
Soils	0.009 to 1.5 mg/kg	Pendias and Pendias, 1992
Plants	0.1-40 mg/kg	Mandal and Suzuki, 2002
Terrestial food	< 1mg/kg	WHO, 1981
Seafood	< 5mg/kg	WHO, 1981
Groundwater	< 10 µg/l	Smedley and Kinniburgh, 2002
River water	0.1-0.8µg/l,	Smedley and Kinniburgh, 2002
Seawater	1.5 µg/l	Smedley and Kinniburgh, 2002

1.2.2 Anthropogenic occurrence of arsenic

Anthropogenic sources of arsenic exceed natural sources by 3:1 (Mandal et al., 2002and reference therein). These consist of insecticides (lead arsenate), herbicides (sodium arsenite, used as weedkiller) desiccants and wood preservatives (chromated

copper arsenate) and feed additives (substituted phenylarsonic acids). Approximately 90% of the world's arsenic production is attributed to China, the USSR, France, Mexico, Germany, Peru, Namibia, Sweden and the USA (U.S. Department of the Interior, 1973; Nelson, 1977). Arsenic can be released into the air, soil and water as a result of human activities such as fossil burning, fertiliser application, the use of arsenical pesticides and the disposal of industrial waste (Mandal et al., 2002). McLeod and co-workers (Bellis et al., 2003) reported arsenic deposited from the atmosphere for the first time on tree bark pockets, a deposition associated with human activities, especially copper industries.

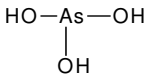
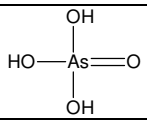
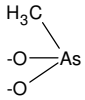
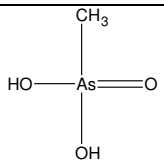
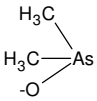
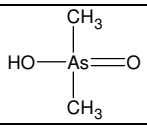
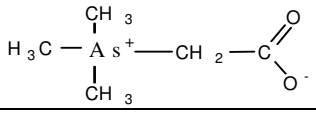
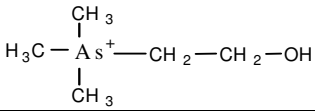
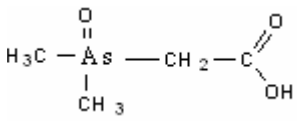
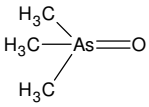
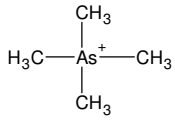
1.3 Arsenic toxicity

The toxicity of arsenic varies due to its chemical forms and oxidation states, in addition to other factors such as physical state (gas, solution or powder). The toxicity of inorganic arsenic compounds decreases in the order AsH_3 > As (III) > arsenoxides > As (V) > arsonium compounds (Mandad et al., 2002; and references cited therein), while that of organic arsenic compounds decreases in the order MA > DMA> MA (Le and Ma, 1997). The toxicity of both arsenic compound forms was reported in increasing order, accompanied by a Lethal Dose of 50 per cent (LD_{50}) as follows: arsenobetaine (AB) < DMA < MA < As (V) < As(III), with corresponding LD_{50} values (mg/kg) in rats of >10000, 700 - 2600, 700 - 1800, 20 and 14 (Le and Ma, 1997). Table 1.3 gives the structures and abbreviations of different arsenic species. Arsenic species such as As (III), As (V), MA and DMA, which are present in natural waters (Le et al., 1994), are more toxic than others which are found in seafood [AB, arsenocholine (AC), tetramethylarsonium ion (TETRA) and arsenosugars] (Samanta et al., 2000; Larsen et

al., 1993). Inorganic arsenic species (As (III) and As (V)) undergo methylation in the human body after ingestion; the major route for elimination of arsenic is urinary excretion (Le et al., 1994). Since the chemical form of arsenic determines its toxicity, it is usually not sufficient just to determine the total level of arsenic in urine; speciation analysis is also necessary.

Ingested inorganic arsenic is metabolised in humans into MA and DMA compounds. Methylarsonite (MA (III)) and dimethylarsinite (DMA (III)) are produced as intermediates during the biotransformation process, with the first of these being 26 time more toxic than As (III) in human hepatocytes (Petrick et al., 2003). MA (III) and DMA (III) were shown to damage DNA in human peripheral lymphocytes, which shows that these species as genotoxic (Mass et al., 2001). Another aspect of arsenic toxicity is inactivation of enzyme systems, which occurs by interaction of As (III) with enzymes through binding to -SH and -OH groups. This interaction mainly favours enzymes with two adjacent SH-groups. The enzymes that generate cellular energy in the citric acid cycle are adversely affected. The inhibitory action involves inactivation of pyruvate dehydrogenase by complexation with As (III), which leads to prevention of adenosine-5-triphosphate (ATP) production. This results in reduction of energy production, and finally slow damage of cells (Mandad et al., 2002). Chronic exposure to arsenic in drinking water results in arsenocosis, the symptoms of which are skin pigmentation, hyperkeratosis of hands and feet (Fig. 1.4), skin cancer, liver damage, peripheral vascular disease, blackfoot disease and diabetes (Shraim et al., 2003; Tseng et al., 1996; Tseng et al., 2004). Acute exposure to arsenic results in vomiting, nausea and garlic odour on the breath (ATSDR, 2000).

Table 1.3 Relevant structures, formulae and molecular weights for commonly occurring arsenic containing compounds found in food and the environment and also detected in human urine.

Compound name	Structure	Formula	Mwt.	pK_{a1}
Arsenious acid (As(III))		H_3AsO_3	126	9.3
Arsenic acid (As(V))		H_3AsO_4	142	2.3
Methylarsonite (MA(III))		$CH_3AsO_2^{2-}$	122	
Methylarsonate (MA)		$CH_3AsO(OH)_2$	140	2.6
Dimethylarsinite (DMA(III))		$(CH_3)_2AsO^-$	121	
Dimethylarsinate (DMA)		$(CH_3)_2AsO(OH)$	138	6.2
Arsenobetaine (AB)		$(CH_3)_3As^+CH_2COO^-$	178	2.2
Arsenocholine (AC)		$(CH_3)_3As^+(CH_2)_2OH$	165	
Dimethylarsinoyl acetic acid (DMAA)		$(CH_3)_2AsOCH_2COOH$	180	
Trimethylarsine oxide (TMAO)		$(CH_3)_2AsO$	136	
Tetramethylarsonium ion (TETRA)		$(CH_3)_4As^+$	135	

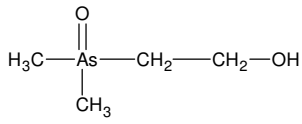
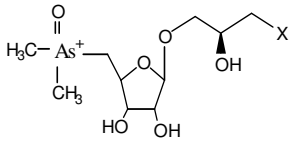
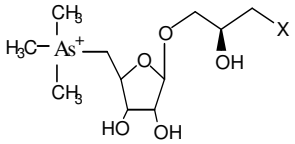
Dimethylarsinoylethanol (DMAE)		$(CH_3)_2AsOC_2H_4OH$	166	
Arsenosugars: Dimethylarsinoylriboside derivatives	 X= SO_3H, OSO_3H, OH, $OPO_3HCH_2CH(OH)CH_2OH$		311+ X	
Arsenosugars: Trimethylarsonioriboside derivatives	 X = SO_3H, OSO_3H, OH, $OPO_3HCH_2CH(OH)CH_2OH$		310+ X	

Figure 1.4 Long-term effect of arsenic in drinking water on feet and hands, modified from Harvard (2007).

1.4 Arsenic metabolism in humans

Humans are exposed to arsenic through drinking water, food or general environmental contamination (Mandal et al., 2002). After exposure, inorganic arsenic in particular will be metabolised into methylated arsenic species. Methylation of arsenic in humans is generally thought to involve alternating reduction and oxidative methylation steps (Cullen and Reimer, 1989). Different methylated arsenic compounds are formed as shown in Fig.1.5 (Challenger, 1945), which describes arsenic's methylation process. Buchet and Lauwerys (1985, 1987) report that Glutathione (GSH) is essential in reduction, and that *S*-adenosyl methionine (SAM) donates a methyl group to arsenic in its trivalent form (in oxidative methylation step) which is sequentially methylated to form MA and DMA. However, Hayakawa et al. (2005) recently reported that the metabolic transformation of As (III) to MA and DMA can take place through a new metabolic pathway involving arsenic methyltransferase (Cyt19) via arsenic triglutathione (ATG) and monomethylarsonic diglutathione (MADG), instead of oxidative methylation of As (III) and MA(III) via the Challenger pathway. The authors suggested that ATG can be generated non-enzymatically from As (III) in the presence of GSH., The methylation of arsenic is subsequently catalysed by Cyt19, a process which involves the transfer of a methyl group from S- SAM to arsenic in the presence of ATG and the formation by MADG of a substrate of Cyt19 for further methylation to dimethylarsinic glutathione (DMAG). These compounds (MADG and DMAG) are oxidised to MA and DMA respectively, which are found as major arsenic metabolites in human urine, as shown in Fig. 1.6 (Hayakawa et al., 2005).

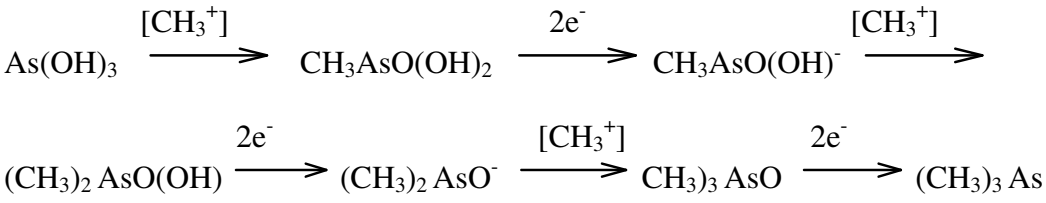


Figure 1.5 Proposed mechanism for the methylation of arsenic (Challenger, 1945)

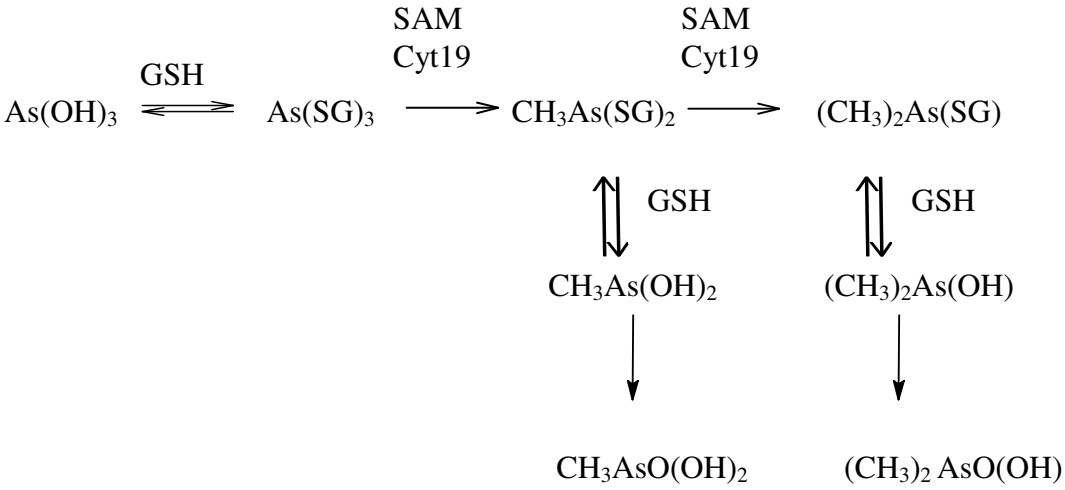


Figure 1.6 A new pathway for the methylation of arsenic (Hayakawa et al., 2005).

The methylation process is generally considered to be a detoxification process, because the MA and DMA produced from the methylation process are less toxic than inorganic arsenic (Vahter and Marafante, 1983a; Fischer et al., 1985). However, other intermediates in the pathway - namely MA (III) and DMA (III) - are now known to be more toxic than inorganic arsenic (Styblo et al., 2000). Therefore, Petrick et al., (2000), have questioned the detoxification role of the pathway. DMA has been reported as a

major metabolite after the ingestion of arsenosugars, which are present in seaweed. This has led to the conclusion that consumption of the arsenosugars could pose a risk to human health (Francesconi et al., 2002; Hulle et al., 2004). Many studies of arsenic speciation have focused on the separation/quantification of As (III), As (V), MA, DMA and AB. The separation of these arsenic species is pH dependent with the pKa_1 values 9.3, 2.3, 2.6, 6.2 and 2.2 respectively (Wrobel et al., 2002) (Table1.3). A simplified schematic diagram indicating the course of arsenic in humans, from intake to excretion (in urine), is shown in Fig.1.7. This figure lists the most commonly found arsenic species in human urine and shows the main biotransformation steps involved in human arsenic metabolism.

1.4.1 Arsenic metabolism in relation to various parameters

Arsenic metabolism is modulated by various biological factors such as exposure to inorganic arsenic through drinking water, dietary arsenic, genetics and ethnicity. Furthermore, levels of certain metabolites and metal ions are also known to modulate arsenic metabolism. Some of these factors are discussed below.

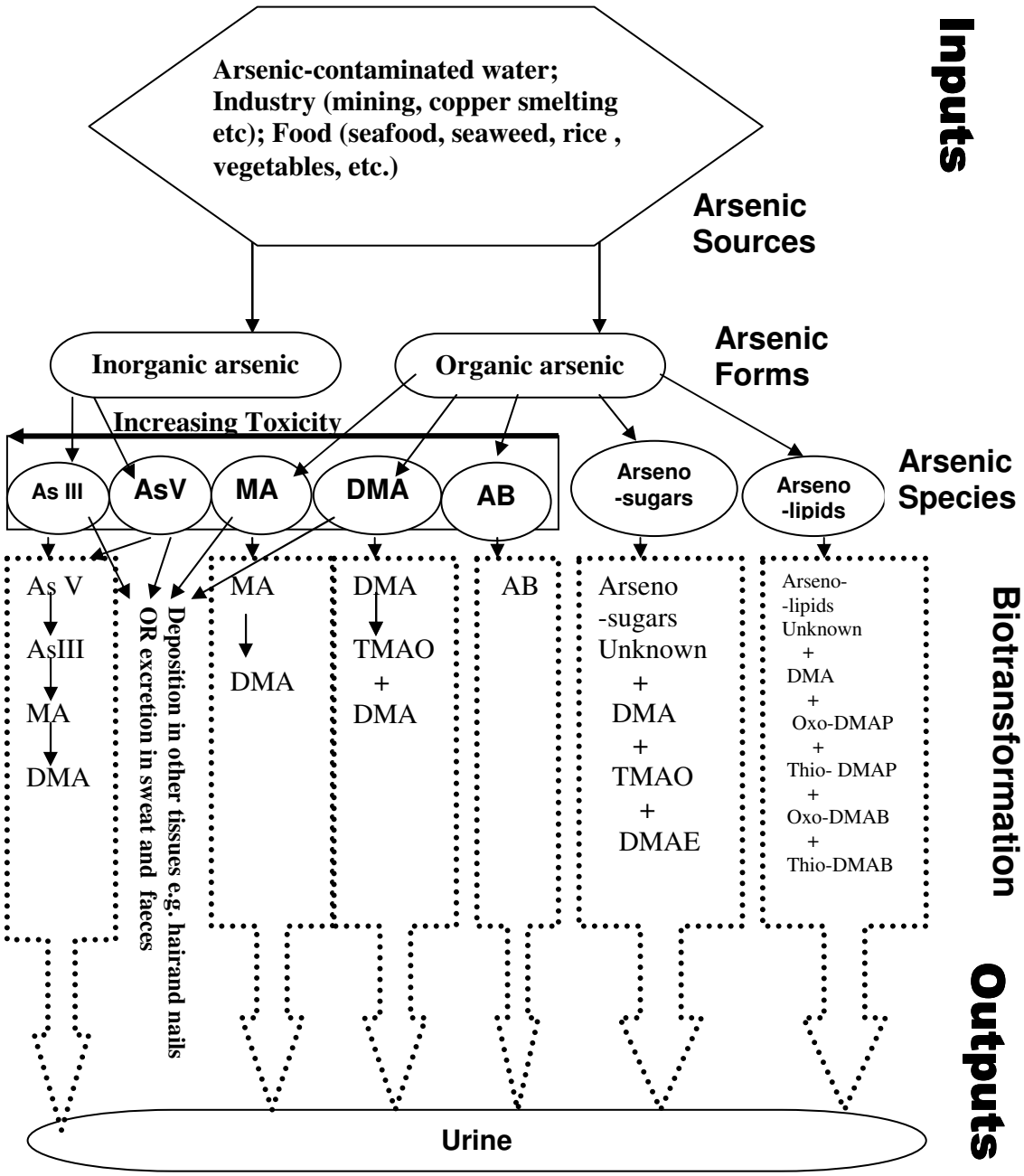


Figure 1.7 A simplified flow chart describing the fate of arsenic in humans. The flow chart only focuses on urinary arsenic excretion.

1.4.1.1 Definition of exposed and unexposed volunteers

Due to its ubiquitous presence in the environment, it is virtually impossible to avoid exposure from arsenic. Hence, all humans are exposed to background levels of arsenic through their diet, drinking water…etc., and as mentioned previously (see Table 1.2) the average concentration of arsenic in the whole human body is 3-4 mg and 0.3-147µg/g (dry weight), with the exception of teeth, hair and nails (WHO, 2001). However, there are situations where humans are exposed to high levels of arsenic. This includes exposure through environmental or occupational sources. These people are often described in the scientific literature as exposed populations. People who are only exposed to background levels of arsenic are often described as unexposed individuals (Ritsema et al., 1998), in order to distinguish them from exposed populations. The term exposed is used either alone or in combination with the words environmental or occupational (Chakraborty et al., 2006; Morton and Mason, 2006; Christian et al., 2006). In this thesis, all the volunteers were only exposed to background levels of arsenic and are therefore termed as unexposed. The term unexposed is used in order to distinguish them from people who are exposed to high levels of arsenic through drinking water, diet or occupational exposure.

1.4.1.2 Arsenic metabolism as a function of exposure

Exposure to arsenic in drinking water is a major area of research. Many studies have been carried out with exposed populations in different countries with high levels

of arsenic in their drinking water, especially Bangladesh, India, Argentina, Taiwan and Chile (Tokunaga et al., 2002; Chowdhury et al., 2003; Vahter et al., 1995; Chung et al., 2002; Lin and Chiang, 1985). The mechanism underlying the presence of arsenic in groundwater in West Bengal and Bangladesh is unclear, although recent studies suggest metal-reducing bacteria may play a role (Islam et al., 2004; Gault et al., 2005b). Different arsenic species were reported in the urine of populations exposed to arsenic in drinking water (mainly inorganic arsenic), with percentages of commonly found species being 60-80% DMA, 10-20% MA and 10-20% inorganic arsenic (Vahter, 1994; Feldman et al., 1999). Some studies investigating exposed populations have not adequately controlled the diet of the volunteers, which raises doubts regarding the distribution of arsenic species detected in urine (Chowdhury et al., 2003). Inhalation was also considered as a route of exposure to arsenic: in a single case in a laboratory a human inhaled trimethylarsine (TMA) during a synthesis process. The inhaled TMA was believed to be metabolised to AB in the human body (Goessler et al., 1997), because a higher level of AB than the background level was detected in urine after exposure to TMA. This however has been questioned by others (Edmonds, 1998). Based on animal experiments, the administered TMA was metabolised to TMAO (Yamauchi et al., 1990), and administered TMAO was excreted unchanged (Yamauchi et al., 1990); there was no evidence of TMAO conversion to AB. Therefore, Edmonds (1998) suggested that the volunteer probably ingested AB-contaminated food.

1.4.1.3 Understanding arsenic metabolism through ingestion studies

In order to understand arsenic metabolism in humans, ingestion studies have been carried out using food rich in arsenic such as seafood and seaweed. In addition to AB, which is the predominant arsenic species in seafood (Ritsema et al., 1998), a substantial increase in DMA was observed in urine, resulting from the metabolism of arsenosugars in seaweed and mussels. The presence of inorganic arsenic and DMA can arise from food sources: Williams et al. (2005) reported significant quantities of these species in rice. Moreover, experimental ingestion studies reveal individual intervariability in arsenic metabolism (Lai et al., 2004). One of the main problems with such experimental ingestion studies is a concern regarding consumption of arsenic-rich food by volunteers. Therefore, these studies are often carried out with a small number of volunteers (5-10), making it difficult to attain reliable conclusions. Generally, experimental ingestion studies with toxic arsenic compounds are carried out with animals. However, Buchet and Lauwerys (1994) reported an ingestion experiment involving 34 volunteers where 500 µg As as inorganic arsenic, MA and DMA were administered as a single oral dose. Their results confirmed that no arsenic species other than inorganic arsenic, MA and DMA were present in urine. This was alongside a partial methylation of inorganic arsenic to MA and DMA, and methylation of MA to DMA, while DMA was excreted unchanged. In another DMA ingestion study in humans, the ingested DMA was equivalent to 0.1 mg As/kg body weight. However, Marafante et al. (1987) reported that 4% of the ingested DMA was excreted as TMAO in urine.

1.4.1.4 Arsenic metabolism as a function of ethnicity/genetics

The role of genetics in arsenic metabolism is still at its infancy, and very few studies have been carried out in this field. However, several genes related to arsenic metabolism have been identified, such as the glutathione S-transferase (GST) genes (for example GSTM1 and GSTP1 null genotypes, which are related to the higher percentage of excretion of MA and DMA respectively) (Ricardo et al., 2006; Chiou et al., 1997). In addition, Aposhian and Aposhian (2006) reported that a number of polymorphisms in human GST omega (ω), CYT 19 and purine nucleoside phosphorylase (PNP) are also linked to changes in urinary arsenic species. Some studies indicate that there is a relationship between arsenic metabolism and ethnicity (Loffreddo et al., 2003; Lai et al., 2004; Wrobel et al., 2002). However, this has not been unequivocally proven as most of these studies have been performed among exposed populations using primarily epidemiological data. Some of these studies compare population residing in different countries, which introduces the problem of a lack of control for variables such as diet (Lai et al., 2004).

1.4.1.5 Homocysteine and folate

Biological compounds such as homocysteine and folic acid are reported to be associated with arsenic metabolism (Chung et al., 2002; Gamble et al., 2005). These molecules are important in the context of this study, since Chapter 5 presents data investigating the effect of fasting on arsenic metabolism. It has been reported that levels of these two biological compounds undergo significant changes during fasting. Subjects undergoing fasting demonstrated an increase in levels of folic acid level (Cahill et al., 1998) and a contrasting decrease in levels of homocysteine level

(Aksungar et al., 2005). De Kimpe et al. (1999) reported that homocysteine inhibits arsenic methylation.

1.4.1.6 Selenium

Selenium (Se) is a metalloid element with atomic weight 79, located in group 16 – close to arsenic (group 15) – in the periodic Table (Fig. 1.2). The valence state of selenium can change from -2, through 0, +2, +4 and +6, which are associated with its geologic distribution, redistribution and use (Harr, 1978). The abundance of selenium is estimated to be the 69th common element in the earth's crust with an average content of 0.05 - 0.09 mg/kg (Glover, et al., 1979). Fishbein (1983) reviewed both natural and anthropogenic occurrences of selenium. Selenium is an essential trace element important for human health and is known to be a component of some enzymes such as glutathione peroxidase and iodothyronine 5`-de-ioninase, in addition to selenoproteins (Arthur et al, 1994; Rayman, 1997; Rayman, 2000).

The dietary sources of selenium include a range of different foods such as Brazil nuts, grain, wheat, cucumber, mushroom and shellfish (Ogra et al., 2004). The ingested selenium species (organic and inorganic selenium) are metabolised to selenide, which is then utilised for the synthesis of selenoproteins, or methylated stepwise to methylated selenium metabolites (Suzuki, 2005). The major urinary selenium metabolite is selenosugar (1{beta}-methylseleno-N-acetyl-D-galactosamine). In response to excessive selenium doses, trimethylselenonium (TMSe) is excreted as a urinary metabolite. The normal level of selenium in urine was reported in the range of 10 – 100 µg/l (Wang et al, 2001).

Selenium was reported to have a role in reducing arsenic toxicity, because the two elements act as metabolic antipodes (Hsueh et al., 2003). Animal experiments have revealed antagonistic interaction between selenium and arsenic (review by Gailer (2007)). A new arsenic-selenium compound (Seleno-*bis*(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$) in the bile of rabbits was identified when injected with aqueous selenite (Se (IV)) and As (III) solutions (Gailer et al., 2000).

An association between urinary selenium and arsenic was also reported by Christian et al.(2006). Urinary selenium was related to a higher percentage of DMA and a lower percentage of inorganic arsenic in human urine. Selenium treatment for arsenocosis patients was reported by Wuyi et al. (2001). The results obtained confirmed decreased level of arsenic in the blood, hair and urine of the treated patients.

1.5 Biomonitoring of exposure to arsenic in humans

Biomonitoring is defined as the measurement of trace compounds in humans (Becker et al., 2003). Biomonitoring is carried out by collecting samples such as blood, urine, hair, nails and breast milk in order to detect the occurrence of biomarkers of exposure. The biomarker is defined as an alteration of biochemical processes in cells that can be measured in biological samples or systems. Therefore, biomarkers can be categorised as those of exposure (detection of analyte and its metabolites) and of effect (detection of biological response) (Becker et al., 2003). In this study the biomarker under investigation is arsenic and as previously discussed this can be monitored in human blood, urine, hair and nails (Yoshida, 2004).

1.5.1 Arsenic determination in humans' biological samples

Studies on arsenic levels in humans have been performed on blood plasma, serum, faeces, urine and ectodermic tissues such as hair, nails and skin-scale (Heyndrickx and Parisis, 1984; Shraim et al., 2003; Mandal et al., 2004; Eisler et al., 1994). Table 1.2 summarises the normal levels of arsenic in different biological samples. However, urine samples are most commonly used for biomonitoring of arsenic exposure in humans.

Blood samples: Blood samples have been used as biofluids to assess arsenic exposure (Wu et al., 2001; Hasegawa et al., 2007), as they reflect an individual's arsenic intake. About 90% of arsenic in blood is rapidly cleared (Vahter, 1983b). Nevertheless, arsenic in blood is a useful indicator of continuous arsenic exposure (Morton and Dunnette, 1994). The method of blood sample collection is through venipuncture, which makes it difficult to study populations. However, blood samples are relatively free from exogenous contamination (Yoshida et al., 2004).

Hair Samples: Hair samples have been used as ectodermic tissue for arsenic exposure (Mandal et al., 2003). Arsenic species such as inorganic arsenic and dimethylarsonate are deposited at the root of hair and migrate into hair shafts. Therefore, the amount of arsenic in longitudinal segments of hair shafts reflects the arsenic exposure at the time point when hair is formed (Yoshida et al., 2004). It follows that the total amount of arsenic in hair samples is an indicator of the average arsenic exposure over a significant period of time. Hair samples are prone to exogenous contamination through dust fixation, sweating or washing the hair with contaminated water. The removal of the external contamination selectively is thus not easy without losing some internal arsenic (Yoshida et al., 2004).

Nail samples: Nail samples, either finger- or toenails, have been used as ectodermic tissues for arsenic exposure (Mandal et al., 2003; Karagas et al., 2001; Wilhelm et al., 2005). The nails samples reflect the arsenic burden for three and six months for finger and toenails respectively, since arsenic binds to sulfhydryl groups of keratin, which then shifts to the tips of the nails during the growing period (Yoshida et al., 2004). The nail samples are also prone to possible external contamination (Yohida et al., 2004).

Urine samples: Urine samples are the most commonly used biological samples for monitoring recent arsenic exposure (Calderon et al., 1999; Wilhelm et al., 2005). The collection procedures of urine samples are 24-h and spot urine samples. The latter is preferred by most researchers because the other method is more troublesome to use and the differences between them are minor (Calderon et al., 1999). The spot urine sample adjusted with creatinine is always used as an index of arsenic exposure, in preference to the more difficult 24-h urine sample collection (Yoshida et al., 2004).

1.5.2 Urinary arsenic analysis

As already mentioned above urine is widely recognised as an important biofluid for identification of biomarkers that can be used both to understand biochemical processes and to develop diagnostic tests. The vast majority of studies investigating arsenic in humans have focused on determining total arsenic and arsenic species in urine rather than other commonly used biological samples such as blood, hair or nails. This is because the clearance of arsenic from the blood is very rapid (Chung et al., 2002), and arsenic content of hair and nails is prone to external contamination (Johnson

and Farmer, 1991). In addition, hair and nails represent long-term exposure values during a period of two to five months for nails and 12 to 18 months for hair (Yoshinaha et al., 1990; Nowak and Kozlowski, 1998). Furthermore, analysis of urine samples has the added advantage that it is abundant and easy to collect. Up to 80% of ingested arsenic is excreted in urine after three days (Hulle et al., 2004), making inorganic arsenic in urine samples an important biomarker for determining recent exposure.

1.5.2.1 Collection and storage of urine

Human urine samples are usually collected in polyethylene bottles and stored at 4 and -20 ^{o}C prior to analysis. Previous studies have suggested that storage conditions have an effect on the stability of arsenic species present in human urine. Very few stability studies (Palacios et al., 1997; Feldman et al., 1999; Yoshinaga et al.,2000; Chen et al., 2002) were carried out on the five arsenic species (As (III), As (V), MA, DMA and AB) most commonly found in human urine. Palacios et al. (1997) found that spiked arsenic species (MA, DMA and AB) in urine sample were stable for 67 days at ambient temperature and 4 ^{o}C. Feldmann et al. (1999) concluded that low temperature (4 and -20 ^{o}C) conditions are suitable for storage of urine samples for up to 2 months, without the use of any additives. AB was reported (Feldman et al., 1999) to be very stable for up to 8 months at three temperatures conditions 25, 4 and -20 ^{o}C. In addition, MA and DMA were also reported to be stable for up to 8 months at 4 and -20 ^{o}C storage conditions, while As (III) and As (V) were less stable under these conditions. Furthermore, the four arsenic species (As (III), As (V), MA and DMA) were less stable at 25 ^{o}C for up to 2 months. Chen et al. (2002) investigated the stability of As (III), As (V), MA and DMA species in human urine at -20 ^{o}C and found their levels constant for

up to 6 months. Yoshinaga et al. (2000) carried out a study to investigate the stability of DMA and AB in freeze-dried urine (CRM NIES No.18) for one and two years, respectively by analysing freshly reconstituted samples. The freeze-dried CRM of these species were found to remain stable during storage at 4^0C (Yoshinaga et al., 2000).

1.5.2.2 Arsenic speciation versus total arsenic analysis in urine

Toxicity of arsenic varies with chemical species and chemical speciation analysis is therefore essential in risk assessment (Le and Ma, 1997). Since biomonitoring requires an accurate analytical method, certified reference materials (CRMs) are of crucial importance for evaluating method performance. The methods used for the certification of arsenic in human urine have previously been discussed (Yoshinaga et al., 2000). Total urinary arsenic cannot be used as an indicator of exposure to toxic arsenic species because it overestimates exposure for those who have consumed food containing non-toxic arsenic (e.g. seafood rich in AB) (Kales et al., 2006). However, determination of toxicologically relevant arsenic compounds (As (III), As (V), MA, DMA) in urine can be used as an indicator of exposure to toxic arsenic. When an individual is exposed to As (V), this species will be reduced to As (III), then methylated to MA and DMA sequentially (Zheng et al., 1999). It is also increasingly being realised that DMA (III) and MA (III) are more toxic than As(III) and As(V) (Gong et al., 2001). Table 1.3 shows structures, formulae, molecular weights and pKa values for commonly occurring arsenic containing compounds found in food and the environment and also detected in human urine. In addition, Table 1.4 shows different arsenic species that have been detected in human urine.

1.5.2.3 Spectroscopic techniques for arsenic determination in human urine

In order to determine what species of arsenic occur in urine, different techniques have been employed. Talmi and Bostick (1975) have reviewed methods for arsenic determinations including spectrophotometric, X-ray, electrochemical, neutron activation and atomic spectrometric techniques. The techniques that have mainly been used for the determination of arsenic and arsenic containing compounds involve chromatographic separation prior to elemental detection. High performance liquid chromatography (HPLC) hyphenated with inductively coupled plasma-mass spectrometry (ICP-MS) utilises the powerful separation of chromatography and the lower limit of detection of ICP-MS. Hydride generation - atomic absorption spectrometry / fluorescence spectrometry (HG-AAS/AFS) has been used for the determination of toxic forms of arsenic (As (III), As (V), MA and DMA) in urine, because only inorganic arsenic, methylated arsenic and recently arsenosugars (Schmeisser et al., 2004) can form arsine gas without a decomposition (mineralisation / oxidation) step. Graphite furnace atomic absorption spectrometry (GF-AAS) is commonly used for urinary total arsenic determination; the advantage of this method is the ease of sample preparation. The determination of arsenic by GF-AAS requires the use of a matrix modifier, which increases ashing temperature and enhances the stabilisation of the analyte. Liquid chromatography mass spectrometry (LC-MS) has been used for the determination of organoarsenic species in urine and arsenosugars in oyster (Shimisu et al., 1999; Sánchez-Rodas et al., 2002). Table 1.4 summarises different arsenic species that have been detected in human urine by using the various spectroscopic techniques.

Table 1.4 Arsenic species that have been detected in human urine by using various spectroscopic techniques. The table is not intended as a comprehensive summary of all the studies reported in the literature, but gives examples of selected applications.

Detection	**Species identified**	**Ref.**
ICP-MS	Total arsenic	(Nixon and Moyer, 1996)
ICP-MS	As (V), As (III), DMA, MA	(Todorv et al., 2005)
ICP-MS	As (V), As (III), DMA, MA, AB	(Wrobel et al., 2002)
ICP-MS	AB, AC, TMAO,Me_4As^+	(Larsen et al., 1993)
ICP-MS	As (V), As (III), DMA, MA, AB, AC	(Moldova et al., 1998)
ICP-MS	As (V), As (III), DMA, MA, AB, AC, Me_4As^+	(Le and Ma, 1998)
ICP-MS	DMA, TMAO, oxo-DMAE, thio-DMAE oxo-DMAA, thio-DMAA, thio-arsenosugar	(Raml et al., 2005)
ICP-MS	DMA, TMAO, DMAE,(9 unknown) arsenosugar metabolites	(Hulle et al., 2004)
ICP-MS	Diphenylarsinic acid	(Shibata et al., 2005)
ICP-MS	DMA, Oxo-dimethylarsenopropanoic acid (Oxo-DMAP), Thio-dimethylarsenopropanoic acid (Thio-DMAP), Oxo-dimethylarsenobutanoic acid (Oxo-DMAB), Thio-dimethylarsenobutanoic acid (Thio-DMAB).	(Schmeisser et al., 2006)
HG-AAS	Total arsenic	(Vahter et al., 1995)
HG-AAS	As (V), As (III), DMA, MA	(Heilier et al., 2005)
HG-AAS	As (V), As (III), DMA, MA, AB, AC	(Moldova et al., 1998)
HG-AAS	As (V), As (III), DMA, MA, AB, AC,Me_4As^+	(Le and Ma, 1997)

HG-AFS	Total arsenic	(Le and Ma, 1997)
HG-AFS	As (V), As (III), DMA, MA	(Heilier et al., 2005)
HG-AFS	As (V), As (III), DMA, MA, AB, AC,$Me_4 As^+$	(Le and Ma, 1997)
GF-AAS	Total arsenic	(Torra et al., 2004)
LC-MS	AB, DMA	(Shimisu et al., 1999)

1.5.3 Arsenic determination in human hair and fingernail

This thesis mainly covers the determination of total arsenic and speciation analysis of arsenic in human urine. In addition, a few samples of human hair and fingernails were analysed for total arsenic, in order to attain a better understanding of the relationship between arsenic metabolism and ethnicity (Chapter 3). The details of the methodology used for hair and nail analysis are provided in Chapter 2.

While urine is considered as a reliable sample for recent arsenic exposure (Wei et al., 2000), hair and nails are considered appropriate for long-term (several months) exposure (Koons and Peters 1994; Takagi et al.1988). Besides the excretion of ingested arsenic through urine, it is also known to be stored in sulfydryl-rich tissue such as hair and nail, being accumulated by binding to sulfydryl groups of keratin in these tissues (Hinwood et al., 2003). External contamination is considered to be a drawback for hair and nail samples, as this could lead to overestimation of internal arsenic or loss of the endogenous arsenic by the washing process (Yoshida et al., 2004). This was confirmed by an absorption experiment reported by Raab and Feldman (2005), which showed that different arsenic species were absorbed by hair. Their study showed that inorganic

arsenic forms As (V) and As (III) are better absorbed by hair than MA and DMA. In speciation analysis of hair and nails of the populations chronically exposed to arsenic, major species are inorganic arsenic accompanied by different amounts of DMA and MA (Mandal et al., 2003).

1.6 Aims of the thesis

This thesis describes the application of different spectroscopic techniques (GF-AAS, ICP-MS and HPLC-ICP-MS) for analysis of arsenic in mainly human urine samples, although some hair and nail samples were also analysed. The aims of the study were to establish a background level for urinary arsenic in unexposed UK population and to explore whether ethnicity and fasting alters the pattern of urinary arsenic excretion. Evaluation of the relationship between arsenic and selenium in human urine were additional aims of the research.

The role of ethnicity in arsenic metabolism in unexposed populations was investigated among volunteers through analysis of biological samples. Urine, hair and fingernails samples of unexposed individuals, from three different ethnic communities residing in the same city (Leicester, UK), were analysed. A possible influence of fasting and the pattern of urinary arsenic were explored by investigating urine samples from Ramadan fasting volunteers. This is important since millions of people in arsenic affected regions of Bangladesh and India perform Ramadan fasting for one month each year. The relationship between arsenic and selenium, which are periodic table neighbours was also investigated by analysis of urine samples from unexposed volunteers. Baseline level of urinary As:Se ratio was determined for these volunteers. This study also involved monitoring urine samples from an individual volunteer over a

period of one year, in order to see whether this ratio alters significantly as a function of diet.

1.7 Summary

Arsenic is ubiquitous in the environment in food and water in organic and inorganic forms, the former being less toxic than the latter. Arsenic is metabolised in humans through a methylation process, in which inorganic arsenic is methylated to MA and DMA. Organic arsenic such as AB is excreted unchanged in human urine. Arsenic exposure in humans can be monitored through analysis of urine, hair and nail samples. Different spectroscopic techniques including GF-AAS, HG-AAS/AFS and ICP-MS have been used for biomonitoring arsenic in such samples. Understanding of arsenic metabolism in humans is still incomplete. Most studies investigating arsenic metabolism have focused on analysis of arsenic in human urine. Such studies fall into the following categories: (i) exposure to environmental and occupational exposure to arsenic (background level, considered as dietary exposure, also being covered); (ii) experimental ingestion studies (the effect of ingesting specific food types - e.g. seafood and seaweed - on urinary arsenic); and (iii) genetics / ethnicity (the role of genetic polymorphism and ethnicity in relation to arsenic metabolism).

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Chapter 2

Instrumentation, Sample Pre-treatment, Experimental Procedures and Method Development

Section 1

2.1 Theoretical background to Instrumentation, Sample Pre-treatment and Chromatography

Different spectroscopic methods were used in this thesis, in order to carry out total arsenic and arsenic species measurement in human urine. Inductively coupled plasma mass spectrometry (ICP-MS) was used throughout this project for total arsenic analysis. High performace liquid chromatography- Inductively coupled plasma mass spectrometry (HPLC-ICP-MS) was used for arsenic speciation measurement in human urine samples. However, hair and nail analysis were carried out using graphite furnace atomic absorption spectrometry (GF-AAS).

2.1.1 Graphite furnace atomic absorption spectrometry (GF-AAS)

The measurement of reduced intensity of electromagnetic radiation from a light source after its passage through a tube containing a gaseous ground state population of atoms is known as atomic absorption. The terms atomic absorption spectroscopy and atoms absorption spectrometry are often used interchangeably, although the former refers to the study of fundamental principles of atomic absorption while the latter refers to use of the atomic absorption for the quantitative determination of elements in samples (Butcher and Sneddon, 1998).

In atomic absorption spectrometry (AAS) the light absorbed by atoms of the analyte is proportional to the number of ground-state atoms present in the atomisation cell (flame or graphite furnace). This has been referred to as Lambert's Law, which can be expressed in the following equation (Moffett, 2000):

A = abc

Where: **A** = absorbance

a = absorption coefficient.

b = length of the absorbance path.

c = concentration of absorbing atoms.

From the above equation it can be seen that, if **a** and **b** are kept constant, then **A** is directly proportional to **c**, which is an ideal case of the Lambert's Law.

In GF-AAS analysis, the sample is heated in a graphite furnace until the element atomises. To analyse arsenic in urine samples, the sample should be diluted and mixed with a matrix modifier, after which the mixture is dried, ashed and finally atomised at an appropriate temperature. The best performance of GF-AAS for arsenic analysis is achieved using a wavelength of 193.7 nm with Zeeman background correction. The splitting and polarisation of atomic spectral lines in the presence of a magnetic field is called the Zeeman effect. The magnetic field does not affect molecules and particulates in the matrix, which cause background absorption. With the magnetic field off, the total absorbance is measured; and with it on only the background is measured (Varian, 2003). A liquid sample (5-100µl) is introduced directly into the tube using an autosampler. The graphite tube is held in place between two electrodes and aligned within the optical path in an AAS instrument, as shown in Fig. 2.1. (Ebdon et al, 1998). Fig. 2.2 shows a picture of GF-AAS (SpectraAA-220Z, Varian, Australia) with

graphite tube atomiser (GTA). The GTA is a device, which is heated to the temperature required for analyte atomisation by passing a current through it. This setup was used for total arsenic determination in biological samples (more details are provided in section 2.2.2).

The graphite tube has disadvantages such as porosity and a tendency for carbide formation. These could be overcome by coating the tube with pyrolytic graphite and by heating the tube in a methane atmosphere. The tube could also be coated with lanthanum to help reduce carbide formation (Ebdon et al., 1998). By programming the power supply controlling the temperature of the furnace, the three steps of drying, ashing and atomisation can be carried out automatically on the sample, each at its particular temperature (Ebdon et al., 1998).

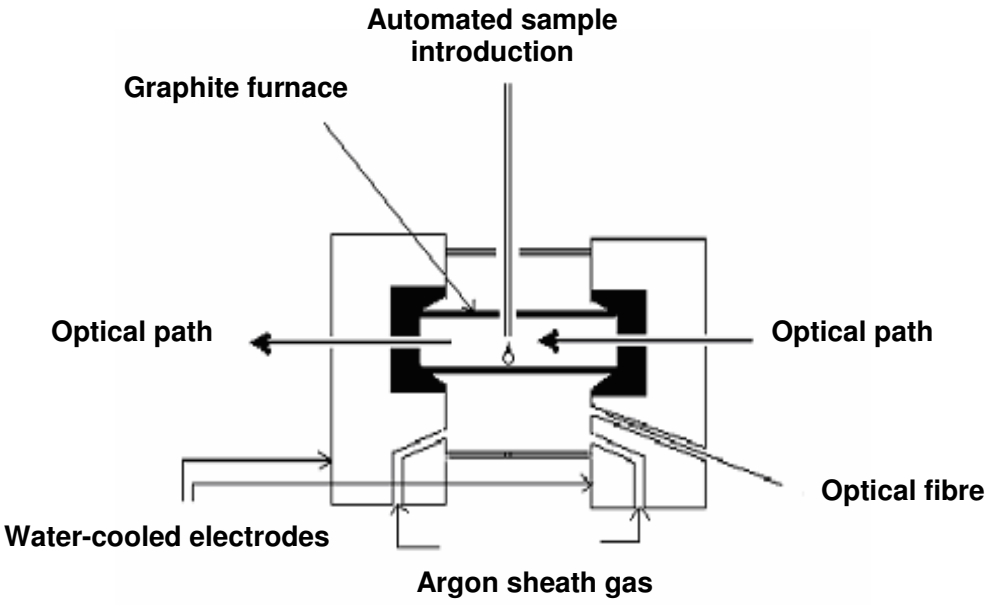

Figure 2.1 Schematic diagram describing the typical setup of a GF-AAS instrument**.**

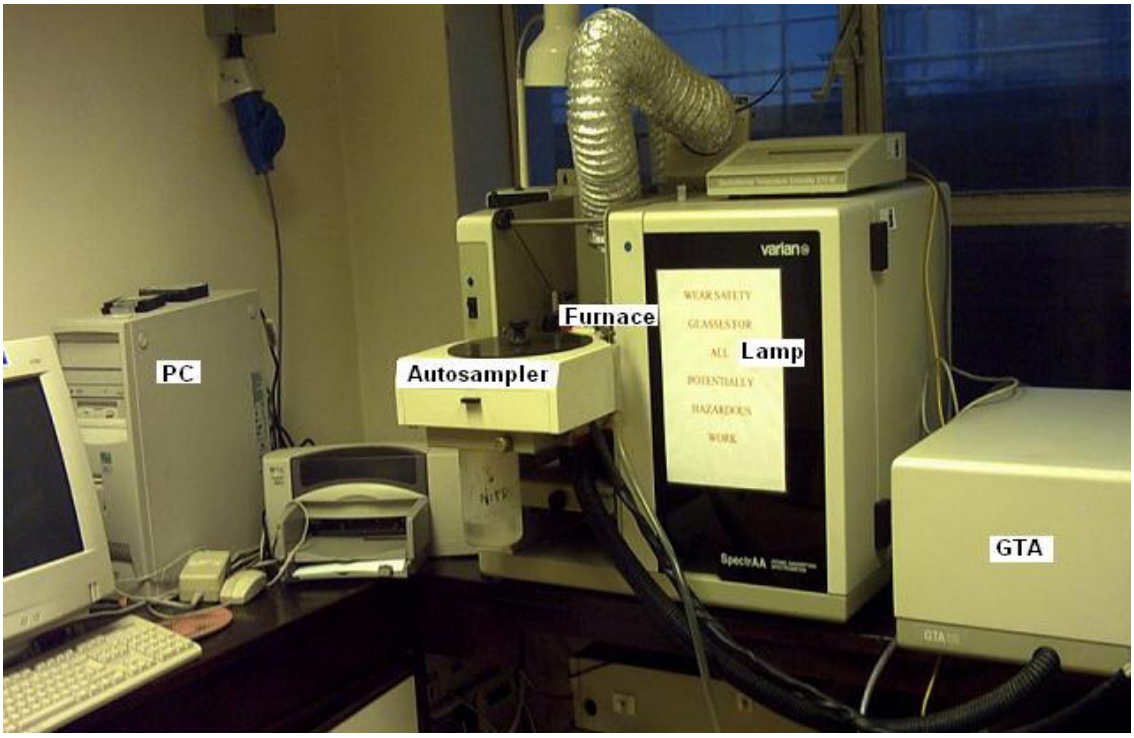

Figure 2.2 Graphite furnace instrument, SpectraAA-220Z with a graphite tube atomiser (GTA), for total arsenic determination in biological samples.

Chemical interference is also one of the disadvantages of the GF-AAS technique and can lead to analyte loss, which occurs when halides are present because of their volatility. It is therefore essential to add a chemical modifier in order to stabilise the analyte during the atomisation temperature by forming intermetallic compounds such as As–Ni. The addition of nitric acid to the urine sample will help form hydrogen chloride (urine contains varying amounts of sodium chloride) (Norymberski and Stubbs, 1956), which will vaporise during atomisation. Background correction is even more essential with furnace atomisation because of the matrix effect, which could be reduced during the ashing step. An inert gas such as nitrogen or argon is used to purge the furnace. Argon is preferred to nitrogen as a purge gas because it has a low diffusion rate, which results in a larger signal, while nitrogen forms nitrides with certain elements such as Ti,

V and Ba. For improved precession and shorter analysis time, the atomiser is cooled with water (Ebdon et al., 1998).

2.1.2 Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS is considered the most efficient technique for elemental analysis of liquid samples. A combination of high performance liquid chromatography (HPLC) with ICP-MS is often the technique of choice in arsenic speciation in human urine. This is because of the ease with which the instrument can be coupled to HPLC and its resolving power, in addition to the sensitivity, lower detection limit and large dynamic range of ICP-MS (Moldovan et al., 1998; Inoue et al., 1994; Samanta et al., 2000).

ICP-MS offers better detection limits for many elements than some other spectroscopic techniques, making it a powerful tool for elemental analysis (Montaser, 1998). The polyatomic interference produced from the chloride ion present in urine is a major drawback of this technique. However, there are new instruments containing reaction cells, which are designed to remove polyatomic interferences (Tanner et al., 2002). Chloride ions react with the argon plasma gas to form argon chloride that has the same mass to charge ratio as arsenic. Five approaches have been used to minimise or eliminate the $^{40}Ar^{35}Cl$'s interference with arsenic. One is the use of hydride generation or chromatography to separate the chloride from the arsenic. A second is the addition of a molecular gas, such as nitrogen, to the plasma to reduce the formation of $^{40}Ar^{35}Cl$. The third is isotopic correction using either an empirically derived relationship of a measured isotope such as $^{35}Cl^{16}O$ with $^{40}Ar^{35}Cl$ or the classical $^{40}Ar^{37}Cl$ / ^{82}Se / ^{83}Kr

correction based on known isotope abundances (Larsen et al, 1993). The mathematical correction for $^{40}Ar^{35}Cl$ is as follows (Gault et al., 2005):

$$I(^{75}As) = I(^{75}As) - R(^{35}Cl/^{37}Cl) \times \{I(77) - R(^{77}Se/^{82}Se) \times [I(82) - R(^{82}Kr/^{83}Kr)]\}$$

where I is the intensity and R is the ratio of natural abundances of specified isotopes.

The fourth approach involves using a high resolution mass spectrometer (sector field double focusing) instead of a quadrupole analyser. The fifth approach offers two possibilities for chemical resolution of interferences: (1) by collision/reaction of the interfering polyatomic ions with gases such as H_2, He or Xe, which destroys a polyatomic ion and consequently removes it from the m/z range of interest; and (2) by reaction of the ion of interest with oxygen, which results in the formation of a new product ion that can be detected at a new non-interfered m/z value (Szpunar, 2005). These five approaches to overcome the polyatomic interferences – chromatography, reaction/collision cell technology, high resolution mass analyser and mathematical correction – have been reported in recent reviews (Szpunar, 2005; Montes-Bayon et al., 2003; Francesconi and Kuehnelt, 2004).

A schematic representation of an ICP-MS is shown in Fig 2.3. In ICP-MS the sample is converted into an aerosol by a nebuliser. Large droplets that may be produced by the nebuliser must be removed in the spray chamber in order to increase precision and to decrease interferences. The aerosol is subsequently transported into the plasma, which usually operates at temperatures of 5000–10000 K at atmospheric pressure. Therefore the ICP is a high energy and hard ionisation source, where the following processes occur sequentially and nearly simultaneously: desolvation-vaporisation-atomisation-excitation-ionisation of the analyte (B'Hymer and Caruso, 2004; Montaser, 1998).

Fig. 2.4 shows a photograph of the ICP-MS (ELAN DRCII, Canada) instrument at Hull University. This setup was used for the determination of total arsenic in human urine (more details are provided in section 2.2.4). Fig. 2.5 shows a photograph of the LC-ICP-MS (PQII, UK) instrument at Manchester University. This setup was used for arsenic speciation analysis in human urine (more details are provided in section 2.2.5).

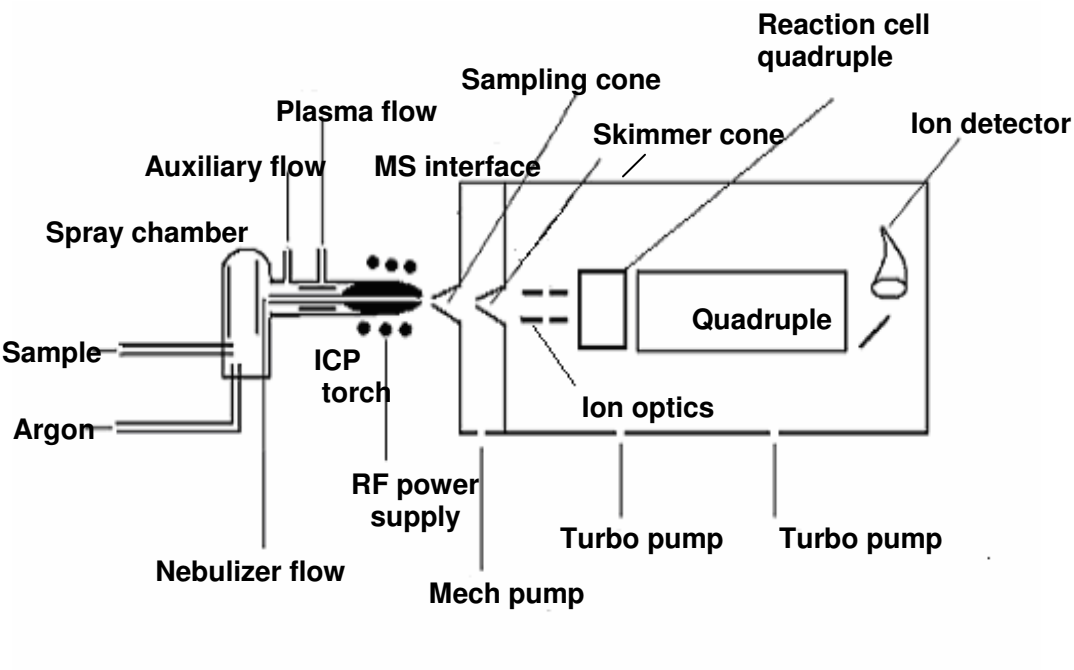

Figure 2.3 Schematic diagram describing the typical setup of the ICP-MS instrument.

Figure 2.4 The experimental setup for total arsenic analysis showing the ICP-MS instrument (ELAN DRCII). This instrument was used at the University of Hull for total arsenic determination in human urine.

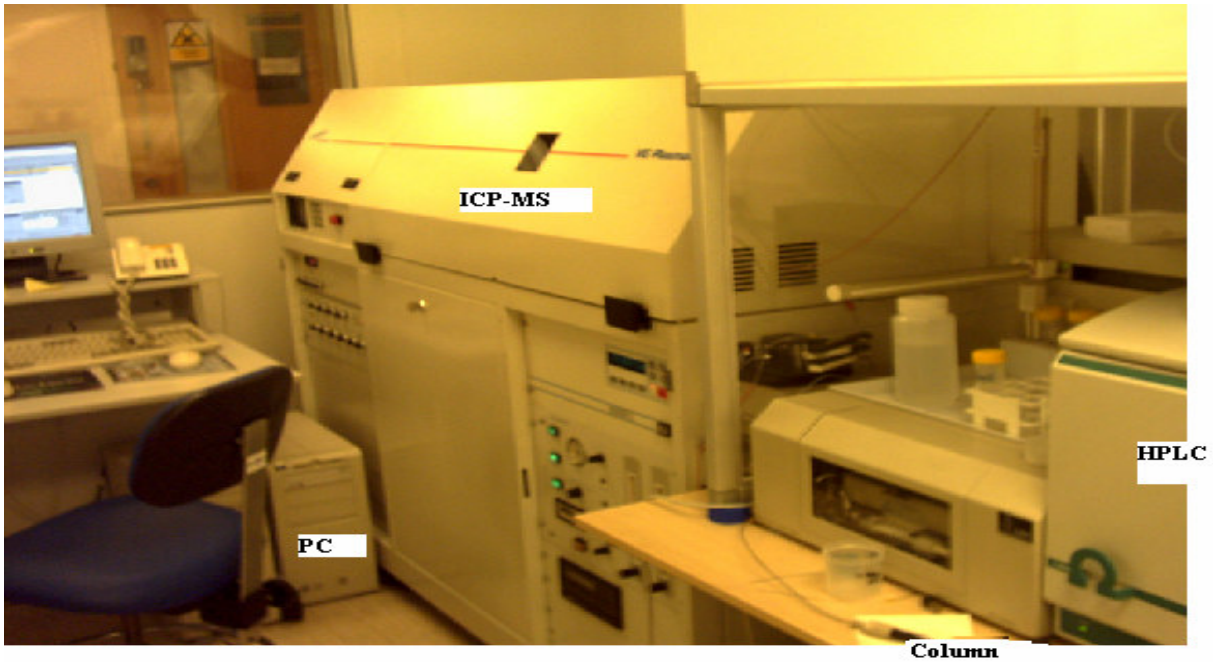

Figure 2.5 The experimental setup showing HPLC pump and column linked to the ICP-MS instrument (PQ II).This instrument was used at The University of Manchester for arsenic speciation in human urine.

In the ICP-MS the end tip of the plasma is extracted into a low-pressure interface containing both a sampler and skimmer cone. The plasma formation takes place in a stream of argon gas flowing through an assembly of three concentric quartz tubes known as a plasma torch (Fig. 2.6). The torch is surrounded at the top by an induction coil connected to a radiofrequency (RF) generator. The induction coil is made of copper and cooled by argon gas or water. A current in the argon gas is induced by a magnetic field generated by the RF through the coil, after the argon gas is seeded with energetic electrons. These electrons are produced either by a high voltage, or a solid state piezoelectric transducer. An efficient introduction of a sample aerosol into a central channel of the plasma results in the efficient desolvation, vaporisation, atomisation, excitation, and ionisation of the sample. A simultaneous multi-element determination can be carried out by argon ICP, since ICP is capable of exciting-ionising a wide range of elements, especially metals. The argon plasma gas isolates and confines an analyte's passageway, which results in sequesteration of the analyte's atomic and ionic species that provides optimum conditions for its sampling from the ICP. The sampling cone orifice of the ICP-MS interface can be easily positioned along the axial channel in a region that leads to maximum analyte signal and minimum background intensity for mass spectrometric measurements. A number of voltages must be set with respect to the ion optics of the mass spectrometer, which in turns depends on the type of ion optics used. Therefore the optimal lens voltage is applied as a function of mass. A quadruple-based mass analyser is used in an ICP-MS instrument because of its relative simplicity, low cost and good performance (Montaser, 1998).

Generally, pneumatic glass concentric nebulisers (PNs) are most commonly used in ICP-MS. PNs are of two basic configurations: concentric or crossflow. In the former, a high speed gas stream surrounds a capillary, through which the sample solution passes. While in the latter the tube carrying a high speed gas stream is set at right angles to the capillary carrying the sample solution. The liquid breaks up because of the mobilisation of a high speed gas, leading to the formation of an aerosol. The analyte transportation efficiency one of the most concerns of the PNs performance is in the range of 1–5%. The PN tends to consume relatively large amounts of sample solution (1–2 ml/min), a problem which low sample consumption nebulisers were developed to overcome (Montaser, 1998). However, the quartz concentric nebuliser provides the lowest possible background signal, an ability ascribed to a minimum background contamination (PerkinElmer, 2004).

There are two main basic types of spray chamber used in ICP-MS. The cyclonic spray chamber is operating by centrifugal force, in which droplets are separated according to size by means of a vortex produced by the peripheral flow of the sample aerosol and argon gas inside the chamber; consequently, smaller droplets are transported with the gas stream into ICP-MS and larger droplets exit through the drain. The other main type, the double pass spray chamber, is commonly used in ICP-MS instrumentation. It is generally of Scott design, in which the selection of the small droplets takes place by directing the aerosol into a central tube, the larger droplets exiting the spray chamber via a drain tube. Pressure in the drain tube forces small drops back between the outer wall side and the central tube, and then they exit the spray chamber into the sample injector of the plasma torch (Thomas, 2001).

The conventional torch that used in ICP-MS has an internal diameter of 18mm and consumes 15 l/min of argon at 1.5kW. The torch consists of three concentric quartz tubes (Fig.2.6): (i) an outer gas flow (12–14 l/min. argon), which sustains the discharge and cools the plasma confinement tube to prevent it from melting; (ii) an intermediate gas flow (0.5 –1 l/min. argon), which is necessary for plasma formation and stabilisation; and (iii) an injector gas flow rate (0.4 – 1 l/min. argon), which carries the sample aerosol into central tube of the torch and plasma. Larger torches are more powerful than smaller torches, because the former operate at higher power levels and gas flow rates, typically providing ICP discharges more robust than the plasma formed in smaller torches. RF is a device used to supply power to ICP discharges, and can be divided into two types: power oscillators (free-running oscillators) and power amplifier/oscillator combinations (crystal-controlled generators). The purpose of the RF is to generate an alternating current at a preferred frequency, using this to ignite and maintain the plasma. As soon as an electrical current passes through the induction coil, the voltage generated across the coil is directly proportional to frequency. The RF generator operates at a frequency range of 2–50MHz, in the majority of ICP instruments typically operating at 27 or 40 MHz with an output power of 1–2kW (Montaser, 1998).

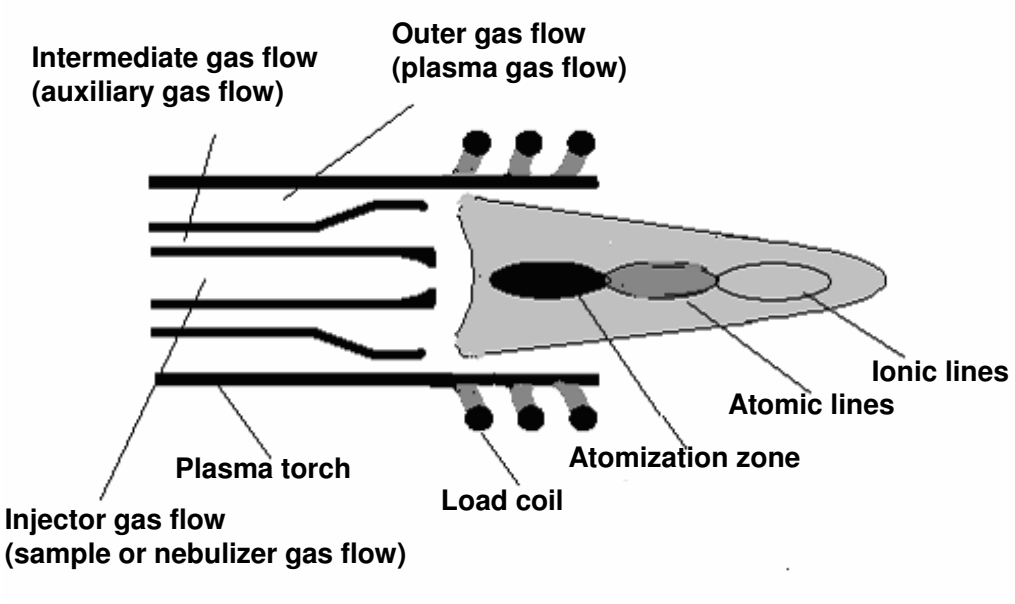

Figure 2.6 Schematic drawing of the plasma torch for ICP-MS (Montaser, 1998).

There are three main fundamental characteristics of the plasma discharges: (i) gas temperature (T_g). (ii) electron temperature (T_e). (iii) electron number density (n_e). Argon ICP has T_g in the range of 4500 – 8000 K , T_e in the range of 8000 – 10,000 K and n_e 1 -3 x 10^{15} cm^{-3}. These conditions combined with relatively long plasma sample interaction time (2 – 3 ms) result in almost complete vaporisation- atomisation of the sample aerosol as well as decreasing of chemical and physical interferences in the plasma. This robustness offers a low-noise condition for detecting a wide range of elements in diverse materials at different concentration (major, minor, trace, and ultra trace) levels (Montaser, 1998).

In order to prevent carbon deposition on the sampler and skimmer cones, it is important to introduce oxygen into the nebuliser gas with an amount in the range of 25-

115 ml/min. A sample with high total dissolved solids can lead to the clogging of the sampler and skimmer cones. Use of a small skimmer cone orifice (~ 0.5 mm) and a relatively large torch injector gas orifice (2.5 mm) combined with a relatively low solution uptake rate (0.4 ml/min.) are capable of reducing the possibility of clogging (Montaser, 1998).

Dynamic reaction cell (DRC) is located between ion optics and the quadrupole (Fig. 2.3). It is function is to remove polyatomic interferences that originate from a combination of plasma gas and sample matrix components before they enter the mass analyser quadrupole. It is unlike the collision cell, which allows all the reaction products to enter into the quadrupole. The DRC uses different reaction gases such as oxygen, methane and ammonia. These reaction gases remove interferences by different processes such as oxidation, electron transfer and proton transfer, collision and dissociation were also considered among these processes (PerkinElmer, 2004).

There are three main types of mass spectrometer used in ICP-MS: magnetic sector, time-of-flight and quadrupole. The commonly used is the quadrupole, which consists of a set of four rods with space in the middle for ions to enter. These rods are connected to each other electrically in opposing pairs. An alternating voltage and RF current are applied to each pair of electrodes. The quadrupole separates ions based on their m/z ratio, allowing only one mass at a given time to pass to the detector (PerkinElmer, 2001).

2.1.3 Sample pre-treatment and chromatographic separation for arsenic in human urine

Matrix effects – particularly in urine samples, which contain a number of different compounds – cause interferences with analyte measurement. Filtration and sample dilution is necessary to reduce matrix effect on the chromatographic separation (Zheng et al., 1999).

Regarding hair and nail samples, the washing procedure is necessary for removing exogenous contamination. The digestion process is an essential antecedent step to the determination of total arsenic in hair and nail samples. The purpose of the digestion process is to destroy most or all the organic matrix of the sample while retaining the analyte element in solution form (Schelkoph and Milne, 1988; Edwards, 1990). These issues are discussed in more details below.

2.1.3.1 Sample filtration

Filtration is normally used in the determination of arsenic in human urine to alleviate the matrix effect. Commonly a 0.45 μm filter is used for filtering the urine prior to total or speciation analysis of arsenic in human urine (Lintschinger et al., 1998; Chen et al., 2002). In addition, urine sample dilution is also essential to overcome this problem (Zheng et al., 1999).

2.1.3. 2 Sample digestion

In general, biological samples such as hair and nails are used as biomarkers for arsenic exposure. These biological samples are always subjected to several pre-treatment steps (sample wash and digestion) prior to analysis. The washing process

involves several steps using 0.1% Triton-X, deionised water and acetone, or ultrasonic, mechanical agitation and deionised water (Schrauzer et al., 1992; Saad et al., 2001; Samanta et al., 2004; IAEA, 1978), in order to remove dirt and any exogenous contamination. The digestion process involves using HNO_3 and H_2O_2 followed by boiling at high temperature or microwave digestion (Mandal et al., 2004; Samanta et al., 2004). Fig. 2.7 shows a microwave (PROLABO A301, France) used for hair and fingernail digestion (more details are provided in section 2.2.2.7).

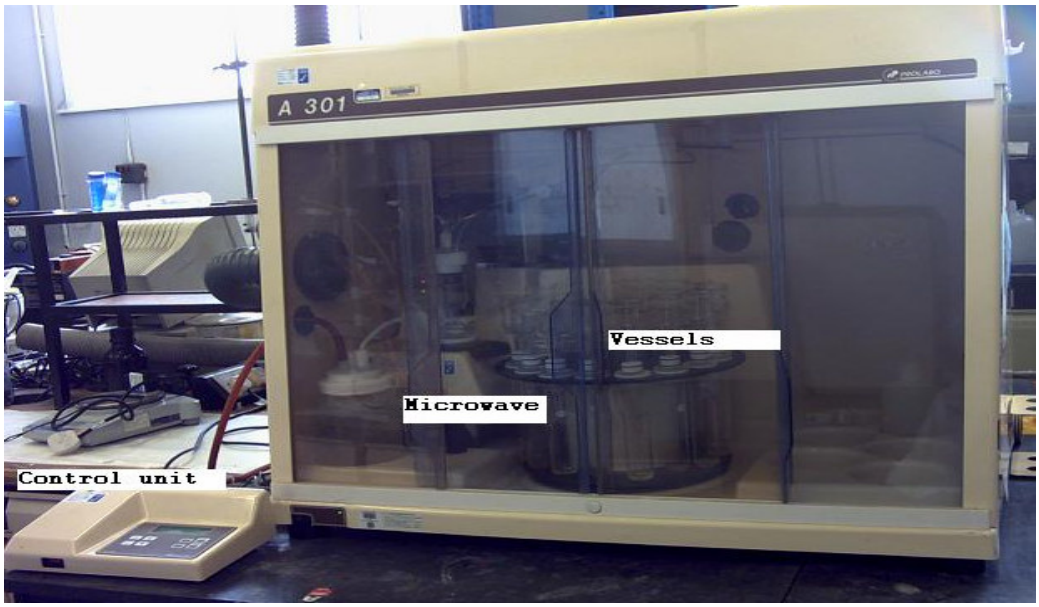

Figure 2.7 Microwave used for digestion of biological samples (hair and nail).

2.1.3.3 Chromatographic separation for arsenic in human urine

For the determination of arsenic species in human urine, different chromatographic separation methods have been employed. These methods have been used to separate arsenic and arsenic containing compounds prior to elemental detection. High performance liquid chromatography (HPLC) hyphenated with inductively coupled plasma-mass spectrometry (ICP-MS), hydride generation atomic absorption

spectrometry (HG-AAS) and hydride generation atomic fluorescence spectrometry (HG-AFS), are the three most commonly used methods for the determination of arsenic and its metabolites in human urine (Lingberg et al., 2007).

Different chromatographic techniques have been used for arsenic separation in human urine such as reversed-phase (RP), reversed-phase ion paring (IP), and ion-exchnage (IEC), which include anion- and cation-exchange chromatography (Shibata and Morita, 1989; Tokunaga et al., 2002; Zheng et al., 1999; Le et al., 2000).

Typically, anion-exchange is the most commonly used method for arsenic speciation (Francesconi and Kuehnelt, 2004) and this approach was used in this study. It is based on the interaction of negatively charged analytes with positively charged functional groups of the stationary phase. The packed materials in the anion-exchange column are beads of crosslinked styrene and divenyl benzene. The ionic functional groups bonded to the packing material are typically quarternary amines or primary amine groups. The mobile phase used in this column is often an aqueous salt buffer mixed with an organic modifier such as methanol (Montes-Bayon et al., 2003). Ammonium carbonate is one of the preferred buffers to be used especially for ICP-MS, because it has shown little signal drift and no excessive residue on the sampler or skimmer cones after prolonged use (B'Hymer and Caruso, 2002; B'Hymer and Caruso, 2004). A photograph showing a typical anion exchange column used during the method development stage of the study is shown in Figure 2.8

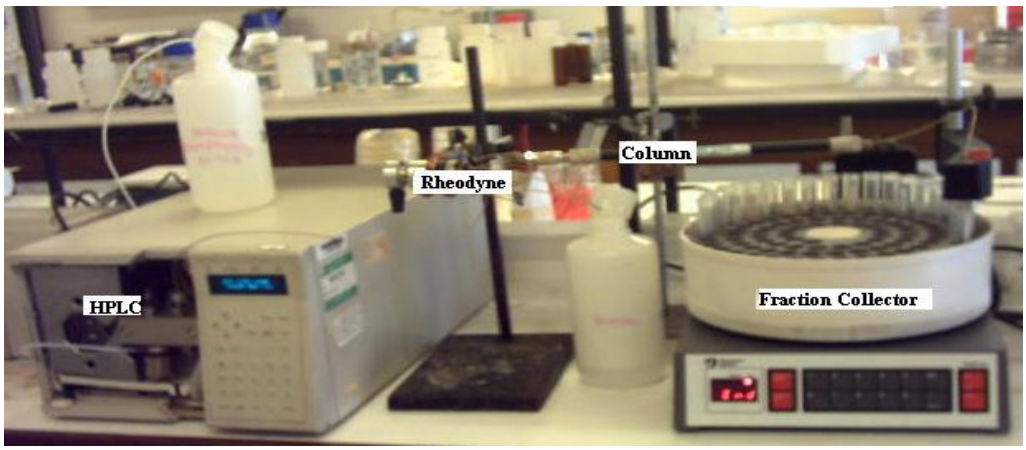

Figure 2.8 The setup of the fraction collector for arsenic species separation using an anion-exchange column chromatographically (PEEK Hamilton, PRP-X100, 4.6 x 250 mm i.d.).

Section 2

2.2 Experimental Procedures and Method Development

This section describes the developed and/or adapted analytical methods used in this study for urinary arsenic analysis. The techniques used were GF-AAS, HPLC-ICP-MS and ICP-MS. Experimental procedures and methods development studies are also presented. It also takes into account the experimental progression employed to optimise the analytical methods by using these techniques. A sub-section on statistical methods applied throughout the thesis is also described.

2.2.1 Methodology

All the steps involved in sample collection, storage, pre-treatment and measurement are discussed in detail in the following sections. There were four techniques used for analysis. GF-AAS was initially used to measure total arsenic in urine. It was also used for off-line speciation analysis. However, this was time-consuming, and no satisfactory resolution for arsenic species was achieved. ICP-MS and HPLC-ICP-MS showed powerful ability to detect both total arsenic and arsenic species, respectively, especially at low levels of arsenic in urine.

2.2.1.1 Methodological steps for arsenic determination in biological samples

As discussed in section 2.1.3, before the urine sample is analysed using spectroscopic techniques it must be subjected to a number of pre-treatment steps. The first step is often filtration, usually through 0.45 μm membrane filter. Subsequently the urine is diluted to alleviate the matrix effect. Thereafter, in the case of speciation analysis HPLC is used to separate the different arsenic species. The sample is then subjected to spectroscopic analysis that identifies and quantifies the different species. The steps involved in urine, hair and nail analysis starting with the collection step and ending with its analysis using different spectroscopic techniques used in this project are illustrated in flow chart in Fig.2.9.

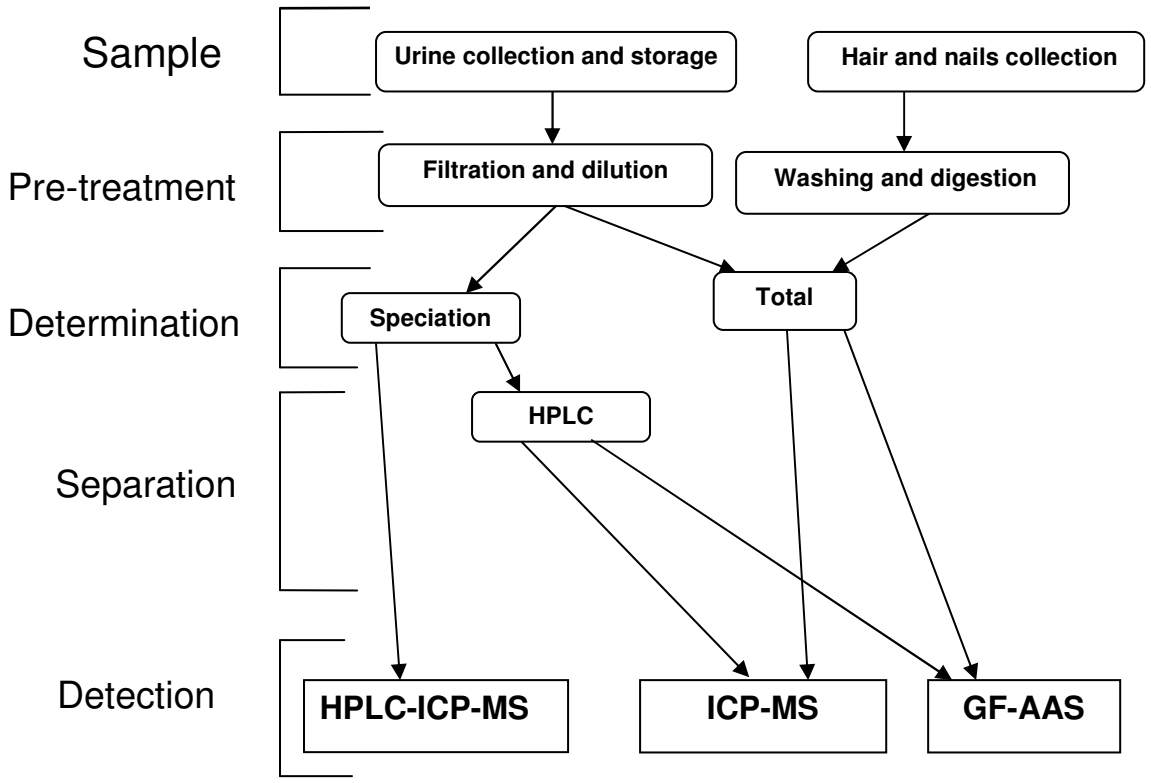

Figure 2.9 Schematic diagram showing the steps used for urine, hair and nail analysis using different spectroscopic techniques.

2.2.1.2 Ethical approval, urine sample collection and preparation

A questionnaire was designed for urine sample collection and was accompanied by a letter to explain to the volunteer what the project was about and how to deal with the sample in terms of collection and storage (Appendix 2.1). The Faculty of Health and Life Sciences, Human Research Ethics Committee, at De Montfort University, Leicester, approved the study design and aims. In the questionnaire, each individual was asked to refrain from eating fish and seafood for 3 days prior to sample collection and to complete a self-administered questionnaire asking for demographic information such as ethnicity, age, gender etc. First morning urine samples (mid-stream) were to be collected directly into polyethylene bottles (Fisher, UK). The pH of each urine

sample was measured using the Combur9Test® (Roche, Germany) urine test strip (Fig. 2.10); nitrite and blood were also measured in each sample. Because dietary nitrate is converted to nitrite by *E .coli* and most urinary tract infections and pathogens, the detection of nitrite is an indication of the presence of nitrite-forming organisms in urine (Roche, 2001). The samples were kept in a freezer at -20 ^{0}C until the analyses were carried out. Prior to analysis, the samples were filtered through a 0.45 µm syringe filter (Millipore, Bedford, USA) and diluted 5-fold with 2 %v/v HNO_3 for total arsenic determination, and 5-fold with the mobile phase for speciation analysis, by using ICP-MS and HPLC-ICP-MS respectively.

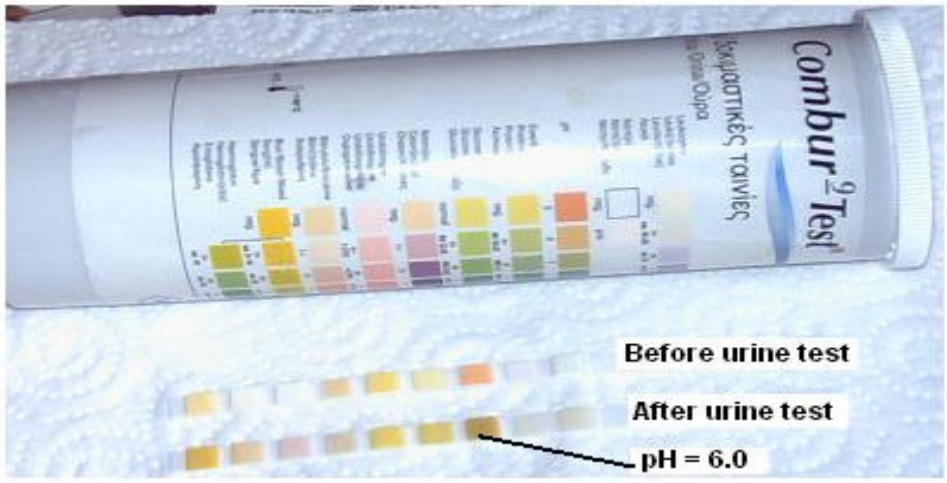

Figure 2.10 Urine test strip to test urine samples for pH, nitrite and blood. The strip is shown before and after the urine test.

2.2.1.3 Determination of creatinine

Creatinine adjustment is routinely used to correct for factors that are not related to arsenic level in urine, such as urine concentration and urine volume (Hinwood et al., 2002). The urine volume can be influenced by fluid intake and respiration rate. Creatinine excretion varies with different factors such as gender, age, body mass and

intake of cooked meat (Hinwood et al., 2002; Carrieri et al., 2001). In population studies investigating environmental arsenic exposure, it is usual to perform a creatinine adjustment (Hinwood et al., 2002).

Creatinine was determined photometrically using a Metra Creatinine Assay Kit (Quidel Corporation, USA), linked to a plate reader measuring absorbance at 540 nm (Multiskan MCC/340, Finland). The principle of the Metra Creatinine assay is based on the Jaffe method, in which a coloured solution results from the reaction between alkaline picrate and creatinine (Quidel, 2004). This assay is quantitative and involves the use of urine samples. The following steps were adopted for the determination of urinary creatinine. Urine samples were diluted with deionised water (1:40), then 150 µl of the working solution was added to the 50 µl of diluted sample after which the mixture was incubated for 30 minutes at room temperature. Finally, the sample was measured by using a plate reader at 540 nm. The calibration graph produced by using a standard solution of creatinine in the range of 0 - 40 mmol/l, is displayed in Fig. 2.11. The measurement of creatinine was validated by measuring a high and a low creatinine standard as shown in Table 2.1.

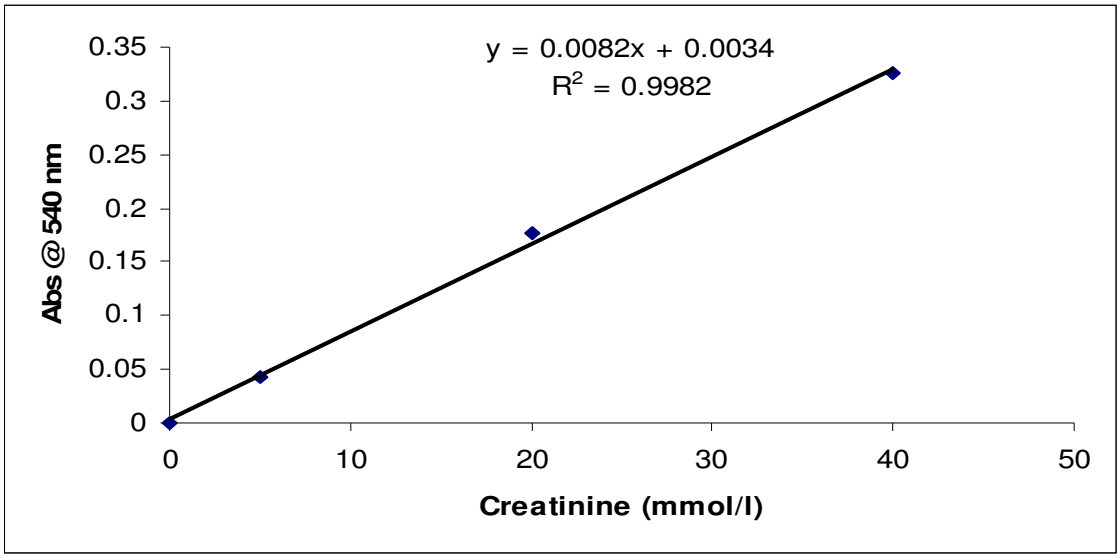

Figure 2.11 Calibration curve for creatinine measurement.

Table 2.1 Concentrations of creatinine standards and their corresponding absorbance at 540 nm.

Creatinine (mmol/l)	Mean Abs. 540nm (n=3)	
0	0	
5	0.043	
20	0.176	
40	0.326	
Creatinine (mmol/l)	Expected average	Found Mean ± SD (n =3)
Control (L)	7.3	7.9 ± 0.0
Control (H)	25.6	24.8 ± 0.1
	Expected range	Found range
Control (L)	6.2 - 8.5	7.9 -7.9
Control (H)	21.5 - 29.6	24.7 - 24.8

2.2.2 Method development for total arsenic using GF-AAS

This section discusses analytical issues associated with the determination of total arsenic analysis. These issues are related to the selection of the chemical modifier, the recovery test, the determination of the limit of detection, and the method of validation. This discussion also includes total arsenic determination in hair and fingernail samples.

2.2.2.1 Chemical modifier selection

The main purpose of using a chemical modifier is to stabilise the analyte and/or to increase the volatility of the matrix. Therefore, most of the matrix can be removed during the ashing (pyrolysis) stage before atomisation, by volatilisation or decomposition (Lima et al., 1998). Nitric acid (UPA, Romil, UK) was used for standards and sample dilution (1% v/v HNO_3) throughout the experiment. Three modifiers were tested:

(1) A palladium modifier (500mg/l) was prepared from Pd (II) acetate (Aldrich, UK) in 0.25% ascorbic acid and 1% v/v HNO_3 (Pohl et al., 1993).

(2) A nickel modifier (1000mg/l) was prepared from $Ni(NO_3)_2.6H_2O$ (BDH, UK) in 5% v/v HNO_3 (Nham, 1989).

(3) A PdS_2O_8 modifier was prepared as a 1:1 mixture of 0.6g Pd(II) acetate (Aldrich, UK) in 15% v/v HNO_3 and an aqueous solution of 2% w/v $K_2S_2O_8$ (Fisher Scientific), (Nixon et al., 1991).

Deionised water (18MΩ/cm, Milli-Q) was used for the preparation of 1% v/v HNO_3, throughout the experiment for sample dilution and standards preparation. Standard solutions of arsenic solution were prepared from a 1000±3 μg/ml As (V) solution (CPI, International) gravimetry.

A SpectrAA-220 GF-AAS system (Varian, UK) with Zeeman background correction was used for the analysis of arsenic in human urine. The instrument comprises a pump system for sample introduction, an autosampler, Zeeman background correction and graphite tube atomiser (GTA) as shown in Fig. 2.1. Table 2.2 gives the temperature programme (recommended by the manufacturer) for graphite furnace measurement of arsenic. The operating conditions of the GF-AAS are shown in Table 2.3.

Table 2.2 Graphite furnace temperature programme for the analysis of arsenic in human urine.

Step	Temperature [C^0]	Time [s]	Flow [l/min.]	Read	Store
1	85	5.0	1.0	No	No
2	95	40.0	1.0	No	No
3	120	10.0	1.0	No	No
4	1400	5.0	1.0	No	No
5	1400	1.0	1.0	No	No
6	1400	2.0	1.0	No	Yes
7	2600	0.6	0.0	Yes	Yes
8	2600	2.0	0.0	Yes	Yes
9	2600	2.0	1.0	No	No

Table 2.3 The operating conditions for GF-AAS

GF-AAS (Zeeman background correction)	
Instrument	SpectraAA-220Z (Varian,UK)
Wavelength	193.7 nm
Lamp current	10 mA
Slit width	0.2 nm
Injection volume	20µl
Modifier volume	2 µl

2.2.2.2 Dilution factor selection

A urine sample from an unexposed volunteer was collected directly into an acid washed polyethylene bottle. The sample was spiked with a known concentration of arsenic (205.8µg/l As (V)) and then diluted to 100, 151 and 201 folds with 1% v/v HNO_3, and then measured by GF-AAS to compare the recovery from different modifiers. The aim was to determine which conditions are suitable for the sample to be accurately analysed. The main variables to be considered were the dilution factor and different the matrix modifiers.

2.2.2.3 Recovery test for the modifiers and dilution factors

%Recovery = 100*(SSR-SR)/SA

where, SSR = spiked sample result

SR = sample result (i.e. unspiked)

SA = spike concentration added

Four calibration standards were prepared for each modifier by using the autosampler automix facility from the As (V) standard (10.34µg/l). The standards 0.00, 1.72, 2.87 and 3.44 µg/l, were used for calibration curves of the modifiers Pd, Ni and

PdS_2O_8. Diluted samples were gravimetrically prepared from spiked sample (205.8µg/l) [(1:100), (1:151) and 1:201)], for recovery test purpose. Recoveries for the three modifiers are shown in Table 2.4.

Table 2.4 Total arsenic concentration (µg/l) in unspiked urine (SR) and spiked urine sample(SSR) and relevant recoveries among the three modifiers

Modifier	Dilution factor	Unspiked urine (µg/l)	Spiked urine (µg/l)	Recovery (%)
Pd	100	<LOD	52.0	25.3
	151	<LOD	79.5	38.6
	201	<LOD	132.0	64.1
Ni	100	110.0	300.0	92.3
	151	148.5	253.5	51.0
	201	168.0	360.0	93.3
PdS_2O_8	100	<LOD	231.0	112.2
	151	<LOD	273.0	132.7
	201	128.0	324.0	95.2

2.2.2.4 The modifier of choice

A 200-fold factor was chosen to eliminate the matrix effect. From Table 2.4 it can be concluded that the greater the dilution, the higher the recovery. The accuracy of this method was established by the quantitative recovery of arsenic added to the urine sample. The test for recovery of arsenic was done by adding arsenic to the urine sample, which was then tested with three modifiers, the best recovery being obtained with the Ni and PdS_2O_8 modifiers at 200-fold. The Ni was preferable to the PdS_2O_8 modifier, and was used as the modifier of choice for economic reasons.

2.2.2.5 Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the equations LOD = 3 x SD; LOQ = 10 x SD (Thomsen et al., 2003), where SD is the standard deviation of ten measurements of the blank (1% v/v HNO_3). Table 2.5 shows the raw data for LOD and LOQ calculations.

Table 2.5 Determination of LOD and LOQ for total arsenic using GF-AAS.

Tube	Sample ID	Conc µg/l	%RSD	Mean Abs	BG Abs	Readings	
1:1	Blk	0.64	22.6	0.0129	0.0190	0.0150	0.0109
1:2	Blk	0.47	21.0	0.0096	0.0157	0.0081	0.0110
1:3	Blk	0.48	25.2	0.0097	0.0126	0.0114	0.0080
1:4	Blk	0.58	8.9	0.0118	0.0165	0.0110	0.0125
1:5	Blk	0.55	12.9	0.0112	0.0188	0.0102	0.0122
1:6	Blk	0.57	17.3	0.0117	0.0201	0.0131	0.0102
1:7	Blk	0.57	21.6	0.0117	0.0232	0.0135	0.0099
1:8	Blk	0.67	23.7	0.0135	0.0132	0.0113	0.0158
1:9	Blk	0.74	21.6	0.0150	0.0159	0.0173	0.0127
1:10	Blk	0.64	0.6	0.0131	0.0140	0.0130	0.0131
Mean		0.591					
SD		0.084					
LOD = 3 x SD		**0.25**					
LOQ = 10 x SD		**0.84**					

2.2.2.6 Method validation

An arsenic standard (30 µg/l) was prepared from As (V) stock solution and used to explore the reproducibility of GF-AAS. This standard was measured ten times, the average was 31.2 ± 1.4 µg/l and the reproducibility was 4.4 %RSD. The typical raw data is presented in the Appendix (2.2). A certified human urine sample (CRM NIES

No.18) was also measured for total arsenic, after a dilution of 200-fold with a blank (1%v/vHNO_3) the result was 147.3 ± 21.2 µg/l (n = 6), compared to the certified result of 137 ± 11 µg/l. Typical raw data for blank, standards, control and CRM are presented in the Appendix (2.2). This study demonstrates that using Ni as a modifier can achieve an accurate measurement of total arsenic in human urine. This method showed that the analysis of arsenic in urine samples can be successfully accomplished by suppressing the matrix interference using high dilution levels. The dilution procedure is simple and efficient. The advantages of this method are a short analysis time, prolonged graphite tube life (at high dilution there are no more residues to degrade the tube) and no pre-treatment of the sample. However, the disadvantage of this method is that it cannot be applied to urine samples with low arsenic concentrations, due to the high dilution factor and the somewhat high LOD of the instrument.

2.2.2.7 Hair and fingernail collection, pre-treatment and total arsenic determination

Hair samples from the head, at different positions, and fingernails, from all the fingers, were collected from unexposed volunteers residing in Leicester, UK. All samples were kept in sealed plastic bags and stored at room temperature. The hair and fingernails samples were washed using the procedure reported by Schrauzer et al. (1992). All samples were washed four times with 1% Triton X-100, then rinsed once with acetone, three times with deionised water, and twice more with acetone. Each sample was washed for approximately 30 minutes and subsequently all the samples were dried in an oven at 70^0C for 20 minutes. An aliquot of 0.06g of fingernails and

0.1g of hair samples were taken for digestion. The digestion procedure was carried out by using 5 ml of concentrated HNO_3 for 20 minutes and 2 ml of 30% H_2O_2 for 10 minutes. Both steps involved application of microwave digestion at 25% power (200W) (PROLABO A301, France) Fig. 2.7. The digested samples were made up to 10 ml in a volumetric flask with deionised water, after which the total arsenic content was determined using GF-AAS instrument.

Certified Reference Material (GBW09101) was used for validation analysis of human hair, which was certified to contain a total arsenic level of 590 ± 70 µg/kg. The mean measured value using GF-AAS was 613 ± 18.4 µg/kg (n =3).

2.2.3 Establishing a method for arsenic speciation in human urine using HPLC-GF-AAS (off-line)

To establish a method for speciation of AB, DMA, MA, As (III) and As (V), the following conditions were used to perform the speciation analysis. HPLC with a fraction collector was setup to perform the arsenic species separation. The chromatographic conditions used are shown in Table 2.6. The separation data of the five standards arsenic species are shown in Table 2.7, and their chromatograms are presented in Fig. 2.12. The aim of this method was to establish arsenic speciation, and to determine the retention time of each species. However, there was a disadvantage of overlap phenomena by using this method. The overlap was observed with the retention times of DMA /As (III) and MA/As (V). This was probably due to the spread of standards over the fractions collection. A complete cycle of one sample separation and

quantification took more than 100 minutes, which was considered as a disadvantage of this method.

A guard column SAX (4 x 3 mm i.d) (Phenomenex, UK) and an anion exchange column PRP-X100 (250 x 4.6 mm i.d) (Hamilton, USA), were used for arsenic species separation. The fraction collector RediFrac (Pharmacia Biotech, Sweden) (Fig. 2.8) was used to collect the fractions. The GF-AAS (Fig. 2.2) system with Zeeman background correction was used for measurement of arsenic in each fraction.

A mobile phase that has been reported previously by Pedersen and Francesconi (2000) was used for arsenic speciation analysis. This mobile phase was slightly modified by using 5% methanol instead of 10%. Yu et al. (2003) reported that the arsenic signal increased with the increase of methanol concentration up to 5%. The 20 mM NH_4HCO_3 mobile phase was prepared by dissolving 1.58 g from ammonium hydrogen carbonate (Fisher Chemicals, UK) in 950 ml deionised water, adjusted with drop-wise addition of 35% NH_4OH solution to obtain a pH 10.3. The mobile phase was then filtered through a 0.45 µm membrane, prior to the addition of 50 ml methanol, and then degassed with helium.

The fraction collector in Fig. 2.8 was operated by the adjustment of fraction collection every one minute; the flow rate of HPLC was 1.5 ml/min. Each fraction volume was 1.5ml. All fractions were collected in tubes and transferred to GF-AAS for total arsenic concentration measurement.

Standard (500 µg As/l) for each species was prepared separately, then a mixture of the five arsenic species (AB, DMA, MA, As (III) and As (V)), all these were prepared in the mobile phase. The speciation process was carried out using the

chromatographic and operating conditions shown in Table 2.6. The data obtained are represented in Table 2.7 and illustrated in Fig. 2.12.

Table 2.6 The chromatographic and operating conditions for HPLC-GF-AAS (off-line).

HPLC	
Column	Hamilton PRP-X100 (250 x 4.6 mm i.d.) PEEK column; guard column SAX (4 x 3 mm i.d.)
Pump	HP 1050 series
Mobile phase	20 mM NH_4HCO_3 + 5%MeOH; pH 10.3.
Flow rate	1.5 ml/min.
Injection volume	100 µl
GF-AAS (Zeeman background correction)	
Instrument	SpectraAA-220Z (Varian,UK)
Wavelength	193.7 nm
Lamp current	10 mA
Slit width	0.2 nm
Injection volume	20 µl
Modifier volume	2 µl

Table 2.7 Fraction time identification for arsenic species (AB, DMA, As (III), MA and As (V)), using HPLC-GF-AAS (off-line).

Standard conc. (µg As/l), separately	Arsenic species	Fraction time (min.)	Measured conc. (µg As/l)
500	AB	2	467.7
		3	28.4
360	DMA	3	337.2
500	As (III)	3	263.4
		4	199.5
		5	48.5
326	MA	7	32.0
		8	139.0
		9	40.0
500	As (V)	11	450.6
		12	106.1
Standard conc. (µg As/l) in mixture	Arsenic species (in mixture)		
500 for each species in the mixture	AB	2	611.1
	DMA	3	598.1
	As (III)	4	42.5
	MA	7	60.8
		8	139.0
		9	40.0
	As (V)	10	54.0
		11	53.0
		12	60.0
		13	20.0

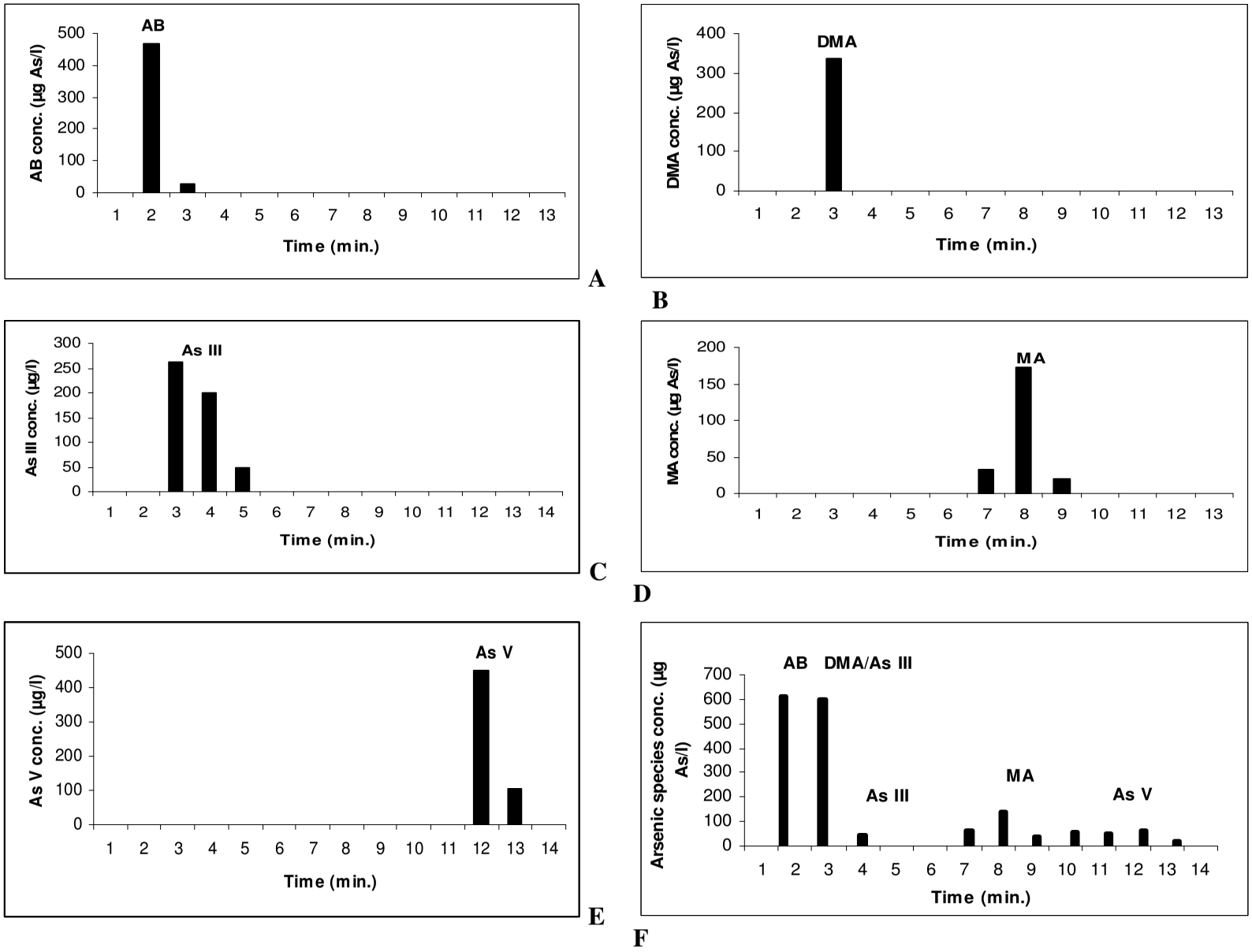

Figure 2.12 HPLC-GF-AAS (off-line) chromatograms of arsenic standards species (A) AB (B) DMA (C) As (III) (D) MA (E) As (V) (F) overlaid chromatograms of the mixture for the five arsenic species.

2.2.4 Method development for total arsenic and selenium in human urine using ICP-MS

A method has been developed for total arsenic analysis in human urine by using ELAN DRCII (PerkinElmer SCIEX, Concord, Ontario, Canada) (Fig 2.4) at The University of Hull; the operating conditions are shown in Table 2.8.

Table 2.8 Operating conditions for ELAN DRCII ICP-MS. A reaction cell was not used; instead, a correction equation was used to remove ArCl interference.

RF power	1350 W
Argon flow rate	
Plasma	15 l/min
Auxiliary flow	1.20 l/min
Nebulizer flow	0.97 l/min
Sample and skimmer cones	Ni
Dwell time	50 ms
Integration time	1250 ms

2.2.4.1 Internal standard selection

The use of an internal standard (IS) is crucial to eliminate volume-related sources of error; this elimination leads to improved precision. This error can occur due to sample solution variability and operating conditions (Altria, 2002). The IS should behave almost identical to the analyte (Nixon and Moyer, 1996). Therefore, the selection of the appropriate IS is important to ensure reliable results. The IS should not interfere with the analyte or be present in the constituents of the sample matrix. Yittrium was chosen as IS after a replicate test of recovery (103%, n = 9) and stability (RSD 2.6%, n = 9). Before Y was chosen, other internal standards (e.g. Gallium,

Indium) were tested, but were shown to have either high recovery or inaccurate results. Therefore, 10 µg/l of yttrium (PlasmaCAL, Québec, Canada) was used as an internal standard for total arsenic analysis.

2.2.4.2 Chloride interference

Human urine contains high NaCl concentrations, hence chloride in the urine matrix interferes with the arsenic signal by forming $^{40}Ar^{35}Cl$, which has the same arsenic nominal mass (75) (Nixon and Moyer, 1996). Interference on monoisotopic ^{75}As by $^{40}Ar^{35}Cl$ was corrected by monitoring the ion counts at m/z = 77, 82 and 83 and applying the correction equation, this based on the equation reported in section 2.1.2, pre-programmed in the ICP-MS software (Elan Instrument Control, version 3.0 hotfix 3). Therefore, the elimination of the $^{40}Ar^{35}Cl$'s interference with arsenic was carried out by the isotopic correction using the classical $^{40}Ar^{37}Cl$ / ^{82}Se / ^{83}Kr correction based on known isotope abundances. An arsenic standard (10 µg/l) was prepared in 400 ppm NaCl solution, in order to check the effectiveness of the equation in removing chloride interference. This effectiveness was confirmed by the recovery of 104.8% (1.1%RSD; n = 3). This proved the efficiency of the equation to remove the interference of the chloride, hence it was applied for the analysis of total arsenic in all samples.

2.2.4.3 Determination of total arsenic in urine samples and method validation

All urine samples were filtered through a 0.45 µm syringe filter and diluted 5-fold with 2 %v/v HNO_3 for total arsenic determination. The use of high purity acid can improve the detection limit and reduce background signal (Mester and Pawliszyn, 2000). The calibration curve of arsenic was drawn within the range of 0 - 20 µg/l (Fig. 2.13). This calibration curve was based on the raw data presented in Table 2.9. The limit of detection (LOD) and the limit of quantification (LOQ) for total arsenic were calculated by measuring the blank (2 %v/v HNO_3) ten times. The LOD (3 x SD) was 0.05 µg/l and LOQ (10 x SD) was 0.18 µg/l. The calculation of LOD and LOQ are shown in Table 2.10. The measurement of total arsenic in urine was validated by a spiking experiment, where the recovery was 103% (n = 9). The accuracy and reproducibility (Table 2.11) of the method were also validated by measuring 10 µg/l standard of arsenic after each 20 runs. The reproducibility (between-run, n =10; replicates (n = 3) for each measurement) was 10.5 ± 0.2 µg/l (1.4% RSD). The spiking experiment was carried out, in order to assess the recovery of total arsenic, by using 50 µg/l of arsenic in urine sample, then diluted (5-fold) with 2%v/v HNO_3 to achieve a total spiked concentration of 10 µg/l. The results are shown in Table 2.12 with a recovery of 104.3% and SD = 2.2. The CRM NIES No. 18 human urine was also used to validate the method and the measured result was 140.7 ± 1.9 µg /l (n = 3); the certified value was 137 ± 11 µg/l.

Table 2.2.9 ICP-MS raw data for total arsenic and selenium in blank, std1, std2, std3, std4 and std5*.

Sample	Element	Mass	Intensity / cps	Sample	Element	Mass	Intensity / cps
Blank	**As**	74.921	**138**	Standard 2	**As**	74.921	**2781**
Blank	Se	76.921	60	Standard 2	Se	76.921	285
Blank	***Se***	81.917	***86***	Standard 2	***Se***	81.917	***358***
Blank	Kr	82.915	91	Standard 2	Kr	82.915	75
Blank	Y	88.905	166445	Standard 2	Y	88.905	159742
Blank	**As**	74.921	**137**	Standard 2	**As**	74.921	**2706**
Blank	Se	76.921	67	Standard 2	Se	76.921	283
Blank	***Se***	81.917	***87***	Standard 2	***Se***	81.917	***347***
Blank	Kr	82.915	87	Standard 2	Kr	82.915	74
Blank	Y	88.905	166300	Standard 2	Y	88.905	164233
Blank	**As**	74.921	**150**	Standard 2	As	74.921	**2747**
Blank	Se	76.921	75	Standard 2	Se	76.921	281
Blank	***Se***	81.917	***74***	Standard 2	Se	81.917	***364***
Blank	Kr	82.915	77	Standard 2	Kr	82.915	81
Blank	Y	88.905	166761	Standard 2	Y	88.905	164595
Sample	**Element**	**Mass**	**Intensity / cps**	**Sample**	**Element**	**Mass**	**Intensity / cps**
Standard1	**As**	74.921	**1452**	Standard 3	**As**	74.921	**7081**
Standard1	Se	76.921	178	Standard 3	Se	76.921	679
Standard1	***Se***	81.917	***225***	Standard 3	***Se***	81.917	***833***
Standard1	Kr	82.915	96	Standard 3	Kr	82.915	86
Standard1	Y	88.905	162740	Standard 3	Y	88.905	164890
Standard1	**As**	74.921	**1412**	Standard 3	**As**	74.921	**6985**
Standard1	Se	76.921	191	Standard 3	Se	76.921	678
Standard1	***Se***	81.917	***245***	Standard 3	***Se***	81.917	***791***
Standard1	Kr	82.915	69	Standard 3	Kr	82.915	74
Standard1	Y	88.905	159961	Standard 3	Y	88.905	166952
Standard1	**As**	74.921	**1500**	Standard 3	**As**	74.921	**6794**

Standard1	Se	76.921	154	Standard 3	Se	76.921	654
Standard1	***Se***	81.917	***221***	Standard 3	***Se***	81.917	***762***
Standard1	Kr	82.915	65	Standard 3	Kr	82.915	85
Standard1	Y	88.905	155595	Standard 3	Y	88.905	169620
Sample	**Element**	**Mass**	**Intensity / cps**	**Sample**	**Element**	**Mass**	**Intensity / cps**
Standard4	**As**	74.921	**13433**	Standard 5	**As**	74.921	**26211**
Standard4	Se	76.921	1225	Standard 5	Se	76.921	2466
Standard4	***Se***	81.917	***1565***	Standard 5	***Se***	81.917	***2968***
Standard4	Kr	82.915	83	Standard 5	Kr	82.915	62
Standard4	Y	88.905	157924	Standard 5	Y	88.905	164711
Standard4	**As**	74.921	**13099**	Standard 5	**As**	74.921	**26491**
Standard4	Se	76.921	1236	Standard 5	Se	76.921	2454
Standard4	***Se***	81.917	***1549***	Standard 5	***Se***	81.917	***2910***
Standard4	Kr	82.915	73	Standard 5	Kr	82.915	83
Standard4	Y	88.905	163278	Standard 5	Y	88.905	164653
Standard4	**As**	74.921	**13311**	Standard 5	**As**	74.921	**26676**
Standard4	Se	76.921	1178	Standard 5	Se	76.921	2376
Standard4	***Se***	81.917	***1489***	Standard 5	***Se***	81.917	***2920***
Standard4	Kr	82.915	65	Standard 5	Kr	82.915	82
Standard4	Y	88.905	165529	Standard 5	Y	88.905	164836

*The blank, std1, std2, std3, std4 and std5 are 2%v/v HNO_3, 1, 2, 5, 10 and 20 µg/l, respectively, for both elements (arsenic and selenium). All arsenic values are in bold and selenium values are in bold and italic. Yittrium (10 µg/l) was used as an internal standard for total arsenic and selenium analysis. As can be seen from above it shows stable intensity (cps) in all standards from blank to std5

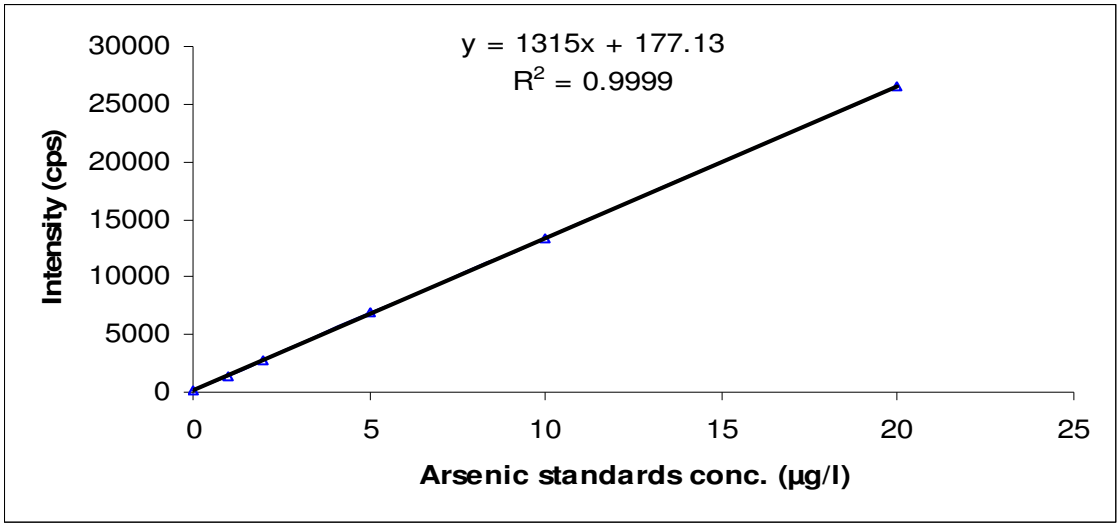

Figure 2.13 Calibration curve for As(V) standards (0,1,2,5,10 and 20 µg/l), by using ICP-MS, under the operating conditions described in Table 2.8.

Table 2.10 Determination of LOD and LOQ for ICP-MS, regarding total arsenic analysis

Sample	**Measured Conc. (µg/l)**	**SD (n = 3)**
Blank-1	0.036	0.038
Blank-2	0.036	0.024
Blank-3	0.010	0.036
Blank-4	0.044	0.040
Blank-5	0.007	0.017
Blank-6	0.043	0.026
Blank-7	0.014	0.009
Blank-8	0.013	0.025
Blank-9	0.039	0.041
Blank-10	-0.004	0.071
Mean	0.024	
SD	0.018	
LOD = 3 x SD	**0.05**	
LOQ = 10 x SD	**0.18**	

Table 2.11 Reproducibility of total arsenic measurement.

Sample	Measured Conc. (µg/l)	SD (n = 3)	%RSD
Standard (10 µg/l)-1	10.721	0.219	2.041
Standard (10 µg/l)-2	10.896	0.265	2.435
Standard (10 µg/l)-3	10.583	0.175	1.657
Standard (10 µg/l)-4	10.533	0.032	0.301
Standard (10 µg/l)-5	10.536	0.170	1.615
Standard (10 µg/l)-6	10.546	0.135	1.279
Standard (10 µg/l)-7	10.585	0.019	0.176
Standard (10 µg/l)-8	10.418	0.150	1.444
Standard (10 µg/l)-9	10.120	0.158	1.557
Standard (10 µg/l)-10	10.090	0.185	1.832
average	**10.5**	**0.2**	**1.4**

Table 2. 12 Spiking experiment to assess the recovery of total arsenic in urine sample.

Sample name	Measured conc. (µg/l) mean ± SD (n =3)	Spiked conc. (µg/l)	Total measured conc. (µg/l) mean ± SD (n =3)	Recovery (%)
US2-1	3.9 ± 0.1	50	54.3 ± 1.3	100.9
US2-2	4.5 ± 0.2	50	56.9 ± 0.2	104.8
US2-3	4.5 ± 0.1	50	56.8 ± 0.2	104.6
Mean				**104.3**
SD				**2.2**

2.2.4.4 Determination of total selenium in urine samples and method validation

A calibration curve of selenium was produced within the concentration range of 0 -20 µg/l (Fig. 2.14). The calibration curve was based on raw data presented in Table 2.13. The limit of detection (LOD) and the limit of quantification (LOQ) for total selenium were calculated by measuring the blank ten times. The LOD (3 x SD) was 0.19 µg/l and LOQ (10 x SD) was 0.63 µg/l (Table 2.13). The spiking experiment was carried out by using 50 µg/l of selenium in urine sample, then diluted (5-fold) with 2%v/v HNO_3 to achieve a total spiked concentration of 10 µg/l. The accuracy and reproducibility of the method was also validated by measuring 10 µg/l standard of selenium after each 20 runs. The reproducibility (between-run, n =10; replicates (n = 3) for each measurement) was 10.3 ± 0.3µg/l (2.6% RSD), and the results are shown in Table 2.14. The measurement of total selenium in urine was validated by a spiking experiment and the recovery was 102% (n = 9) (Table 2.15). The CRM NIES No. 18 human urine was also used to validate the method and the measured result was 67.9 ± 4.5 µg /l (n = 3); the certified value was 59 ± 5 µg/l.

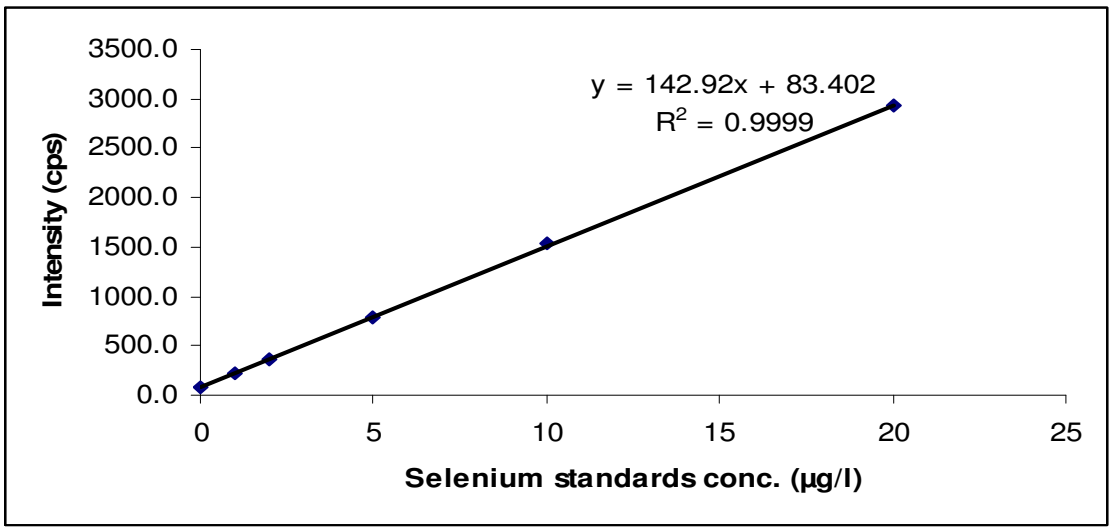

Figure 2.14 Calibration curve for selenium standards (0,1,2,5,10 and 20 µg/l), by using ICP-MS. The operating conditions are described in Table 2.8.

Table 2. 13 Determination of LOD and LOQ for ICP-MS regarding total selenium analysis.

Sample	**Measured Conc. (µg/l)**	**SD (n = 3)**
Blank-1	0.141	0.169
Blank-2	0.064	0.065
Blank-3	0.074	0.034
Blank-4	0.141	0.082
Blank-5	0.040	0.067
Blank-6	0.073	0.047
Blank-7	0.068	0.094
Blank-8	-0.004	0.069
Blank-9	0.109	0.113
Blank-10	-0.064	0.140
Mean	0.064	
SD	0.063	
LOD = 3 x SD	**0.19**	
LOQ = 10 xSD	**0.63**	

Table 2.14 Reproducibility of total selenium measurement.

Sample	Measured Conc. (µg/l)	SD (n = 3)	%RSD
Standard (10 µg/l)-1	10.416	0.25	2.398
Standard (10 µg/l)-2	10.371	0.274	2.639
Standard (10 µg/l)-3	10.720	0.112	1.042
Standard (10 µg/l)-4	10.465	0.108	1.032
Standard (10 µg/l)-5	10.441	0.342	3.277
Standard (10 µg/l)-6	10.387	0.479	4.612
Standard (10 µg/l)-7	10.543	0.392	3.715
Standard (10 µg/l)-8	10.211	0.205	2.009
Standard (10 µg/l)-9	9.670	0.066	0.679
Standard (10 µg/l)-10	9.939	0.470	4.726
Average	**10.3**	**0.3**	**2.6**

Table 2.15 Spiking experiment to assess recovery of selenium.

Sample name	Measured conc. (µg/l) mean ± SD (n =3)	Spiked conc. (µg/l)	Total measured conc. (µg/l) mean ± SD (n =3)	Recovery (%)
US2-1	14.1± 0.1	50	65.0± 1.8	101.8
US2-2	15.1± 0.2	50	67.0± 0.4	103.8
US2-3	15.0± 0.2	50	65.8± 0.3	101.6
Mean				**102.4**
SD				**1.2**

2.2.5 Method development for arsenic speciation in human urine using HPLC- ICP-MS

A method has been developed for determination of arsenic species in human urine samples using ICP-MS. A PQ II VG Instrument (Winford, UK), shown in Fig. 2.5, was used for this speciation analysis. The measurements were carried out at the University of Manchester and the operating conditions are detailed in Table 2.16.

Table 2.16 Instrumental operation conditions for HPLC-ICP-MS system: ICP-MS (PQ II, VG Instruments, Winford, UK).

HPLC	Instrument
Column	PEEK Hamilton PRP-X 100 anion exchange (250 x 4.6 mm i.d).
Guard column	Phenomenex Polymerx RP-1 (4 x 3 mm i.d), pH stability (1-14).
Mobile phase	95% v/v 20 mM NH_4CO_3, pH 10.3 and 5% v/v methanol.
Injection volume	100 µl
ICP-MS	Instrument
Radio frequency power	1335 W
Sample and skimmer cones	Ni
Argon flow rate	
Plasma	13 l/min
Auxiliary flow	0.95 l/min
Nebulizer flow	0.94 l/min
Acquisition parameters	Peak jumping
Dwell time	100 ms
Data acquisition time	1300 ms

2.2.5.1 Chromatographic conditions

Germanium (Ge) solution was added to the mobile phase (see Table 2.16) as an internal standard to a final concentration of 50 µg/l. The HPLC system consisted of a 790 Personal IC chromatograph (Metrohm, Switzerland), a liquid chromatography solvent delivery pump, fitted with a 100 µl sample loop, and an anion exchange column Hamilton PRP-X 100 (250 × 4.6 mm i.d.) with a guard column (4 × 3 mm id), Polymerx RP-1 (Phenomenex, USA). An ICP-MS was used as the chromatographic detector. The outlet of the HPLC system was coupled directly to the inlet of the ICP nebulizer with PEEK i.d. 90 µm tubing. Signals at m/z 75, 77 and 51 were monitored. The signal at m/z 51 was used to monitor the $^{35}Cl^{16}O^{+}$ interference. The chromatographic and instrumental conditions for arsenic speciation are shown in Table 2.16.

2.2.5.2 Speciation analysis of arsenic in urine samples and method validation

HPLC-ICP-MS was used for the separation of five arsenic species: AB, DMA, MA, As (III) and As (V). The calibration curves of the arsenic species were drawn within the range of 0 -25 µg As/l. The calibration curves were based on the raw data presented in Fig. 2.15, 2.16, 2.17, 2.18 and 2.19 for the blank (mobile phase(0)), 1,5,10 and 25 µg As/l standards, respectively. The calibration curves of the five arsenic species are presented in Fig. 2.20. The problem of interference from high levels of chloride present in the urine samples was overcome as shown in Fig.2.21. The chloride was chromatographically resolved from the various arsenic species. Arsenic species peak integration and drift correction were calculated using an in-house Turbo Pascal

programme. Detection limits for the five species (AB, DMA, As (III), MA and As (V)) were 0.45, 0.45, 0.95, 0.84 and 0.99 µg As/l, respectively.

To assess the recovery of the five arsenic species, a spiking experiment was carried out, by using 10µg As/l for each species in a mixture. The results are shown in Fig. 2.22 for unspiked urine sample, and Fig. 2.23 for the same urine sample spiked with the mixture. Furthermore, a control experiment was carried out by spiking a urine sample individually with different species to confirm the retention time of the species in urine samples especially for the DMA and As (III) that eluted close to each other. The result of unspiked urine sample is shown in Fig.2.24, the same urine sample was spiked with 10µg As/l for AB, DMA and As (III) separately, this shown in Fig. 2.25, 2.26 and 2.27, respectively. An overlaid chromatogram for the above-mentioned figures (Fig.2.24 to 2.27) is presented in Fig. 2.28. This shows a recognised peak for each species. The recoveries of the arsenic species spiked in the urine sample were AB (90 %), DMA (95 %), As (III) (86 %), MA (95 %), and As (V) (98 %); results are presented in Table 2.17.

The accuracy and reproducibility of the method was also validated by measuring 10 µg As/l standard mixture of the five arsenic species after each six runs. The results are shown in Table 2.18, and the raw data are shown in Appendix 2.3. The CRM NIES No. 18 human urine was used to validate the method and the results were as follows: AB 69.7 ± 0.3 µg As/l and DMA 38.4 ± 0.4 µg As/l; the certified values were 69 ± 12 µg/l and 36 ± 9µg/l, respectively.

Table 2. 17 The recovery percentage of the five arsenic species spiked in urine sample.

Arsenic species	AB	DMA	As (III)	MA	As (V)
Measured conc. (µg As/l) ± SE	9.0 ± 1.3	9.5 ± 0.1	8.6 ± 1.1	9.5 ± 1.2	9.8 ± 6.4
Spiked conc.(µg As/l)	10.0	10.0	10.0	10.0	10.0
Recovery (%)	90	95	86	95	98

Table 2.18 Reproducibility of the five arsenic species measurement in 10µg As/l standard mixture.

Arsenic species	AB	DMA	As (III)	MA	As (V)
Measured conc. (µg As/l) ± SE (1)	10.0 ± 0.2	9.9 ± 0.3	10.1 ± 0.6	10.1 ± 0.3	10.3 ± 1.1
Measured conc. (µg As/l) ± SE (2)	10.1 ± 0.3	9.9 ± 0.7	10.0 ± 1.4	10.0 ± 0.6	10.8 ± 1.2
Measured conc. (µg As/l) ± SE (3)	9.9 ± 0.4	9.8 ± 0.8	10.2 ± 1.5	10.1 ± 0.7	9.9 ± 1.2
Mean	10.0	9.9	10.1	10.1	10.3
SD	0.1	0.1	0.1	0.1	0.5
%RSD	1.0	1.0	1.0	1.0	4.9

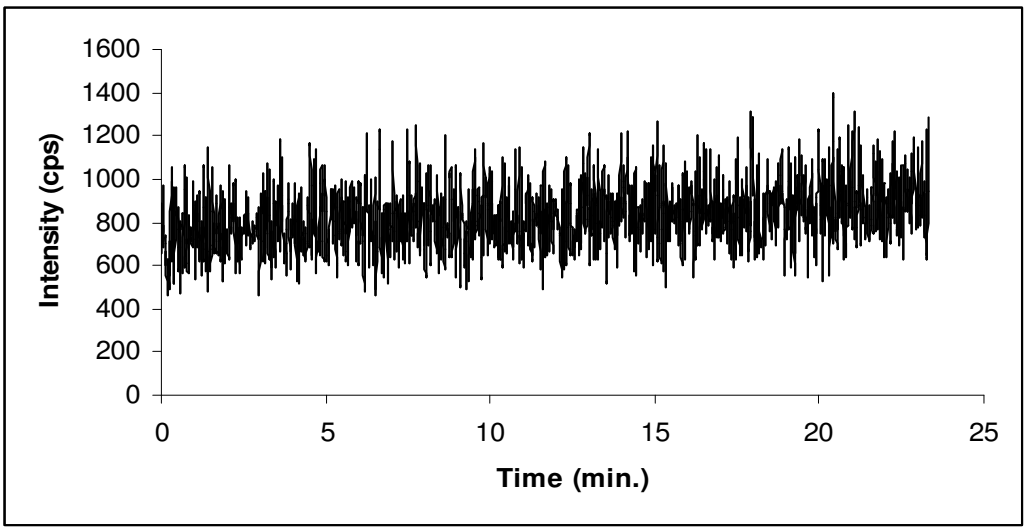

Figure 2.15 HPLC-ICP-MS chromatogram of the blank (mobile phase).

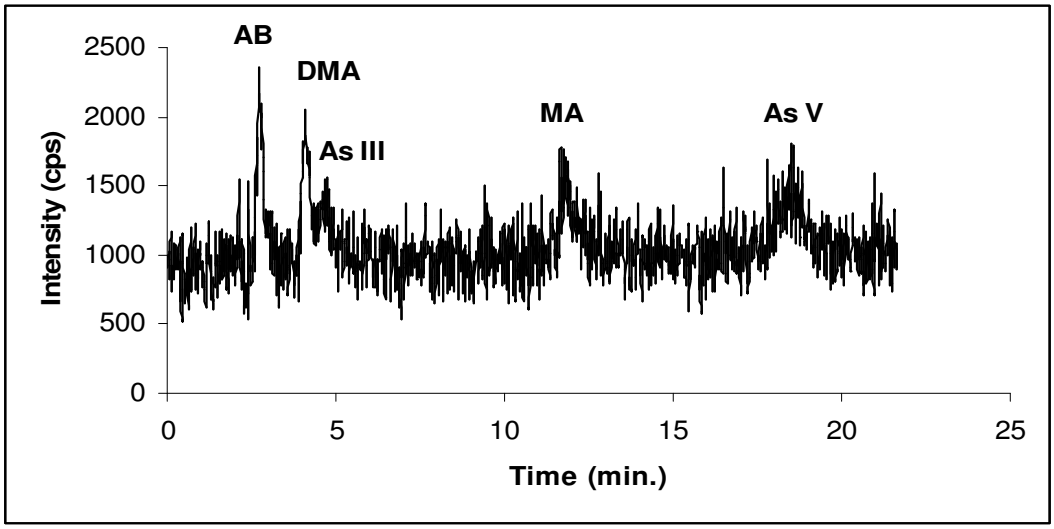

Figure 2.16 HPLC-ICP-MS chromatogram of a standard containing 1 µg As/l of each AB, DMA, As (III), MA and As (V).

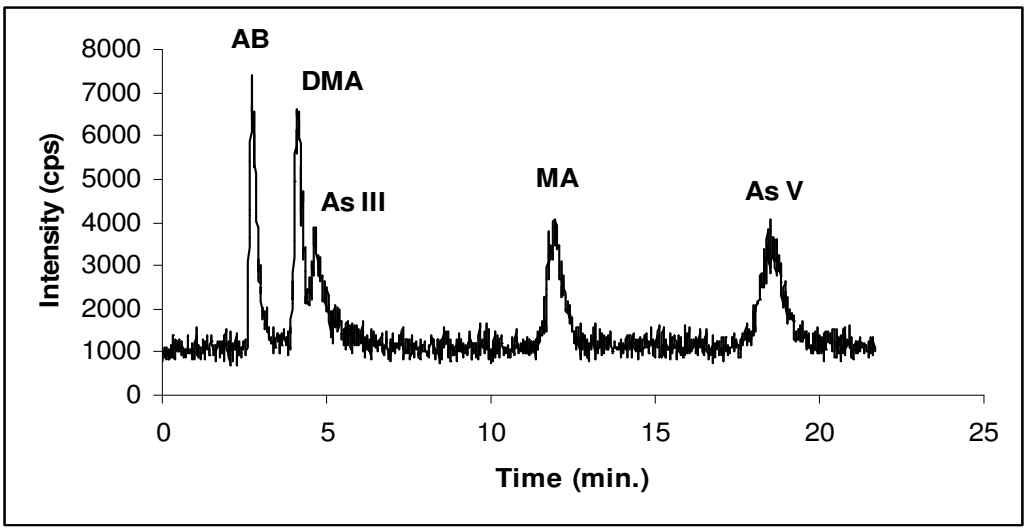

Figure 2.17 HPLC-ICP-MS chromatogram of a standard containing 5 µg As/l of each AB, DMA, As (III), MA and As (V).

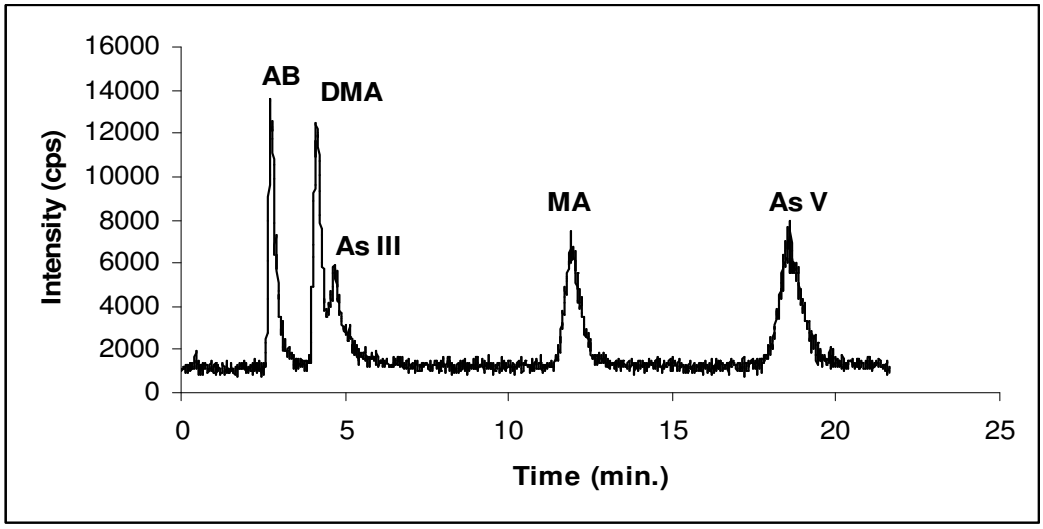

Figure2.18 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l of each AB, DMA, As (III), MA and As (V).

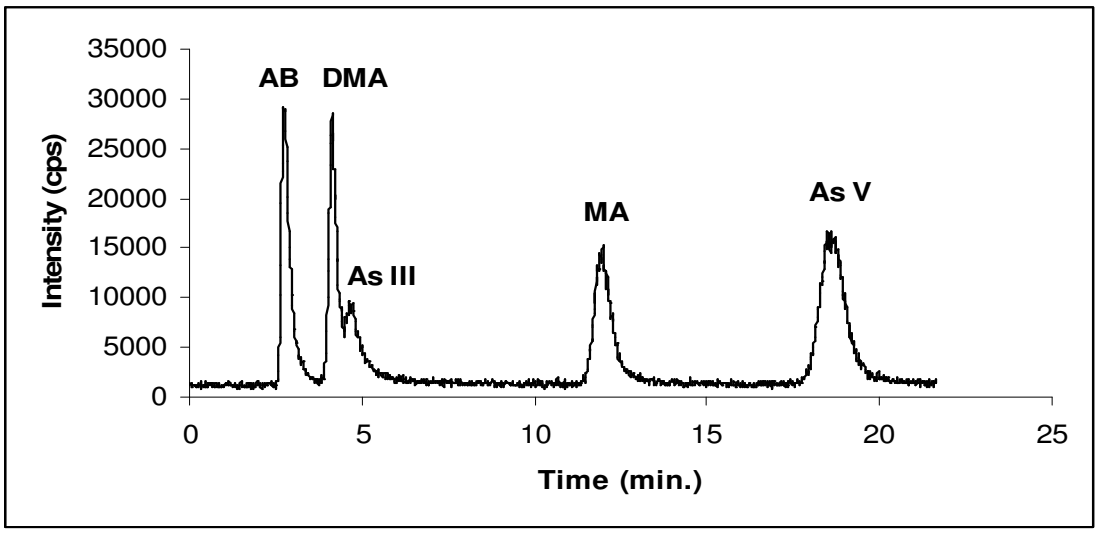

Figure 2.19 HPLC-ICP-MS chromatogram of a standard containing 25µg As/l of each AB, DMA, As (III), MA and As (V).

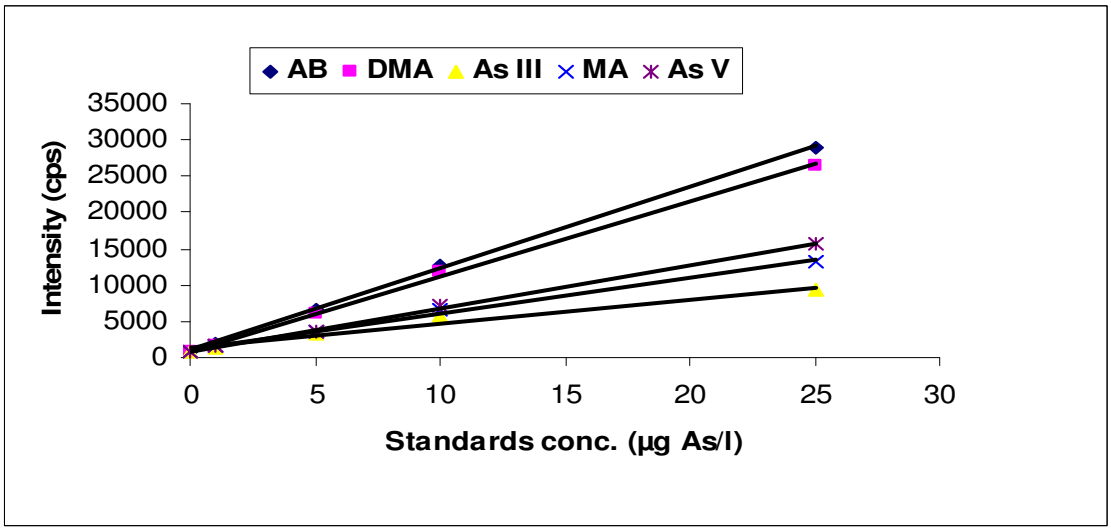

Figure 2.20 Calibration curves for standards arsenic species (AB, DMA, MA, As (III) and As (V)), the range of arsenic concentration is 0 – 25 µg As/l.

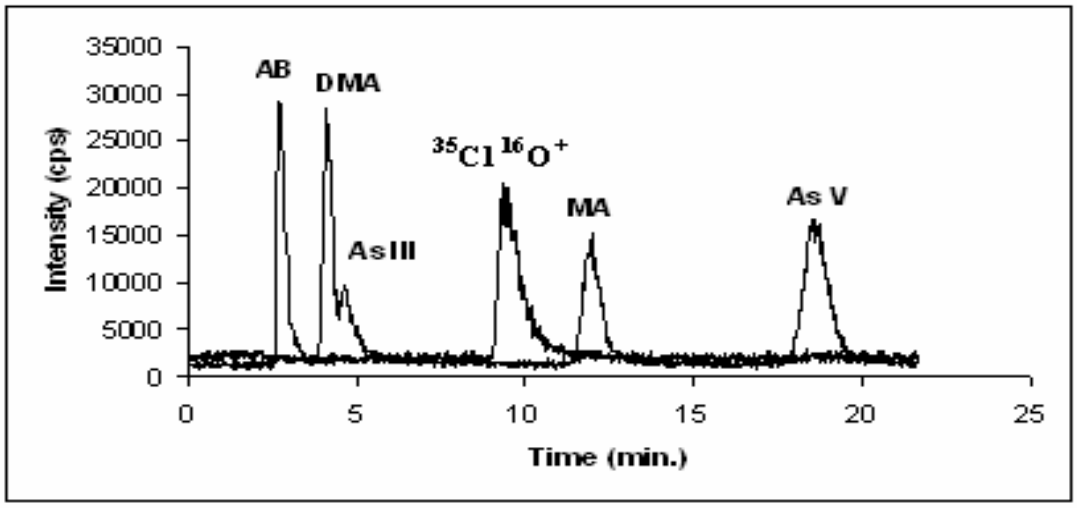

Figure 2.21 Chromatogram of standard solution containing five arsenic species (25 µg As/l) overlaid with $^{35}Cl\ ^{16}O^{+}$ peak from diluted human urine; experimental conditions are given in Table 2.19.

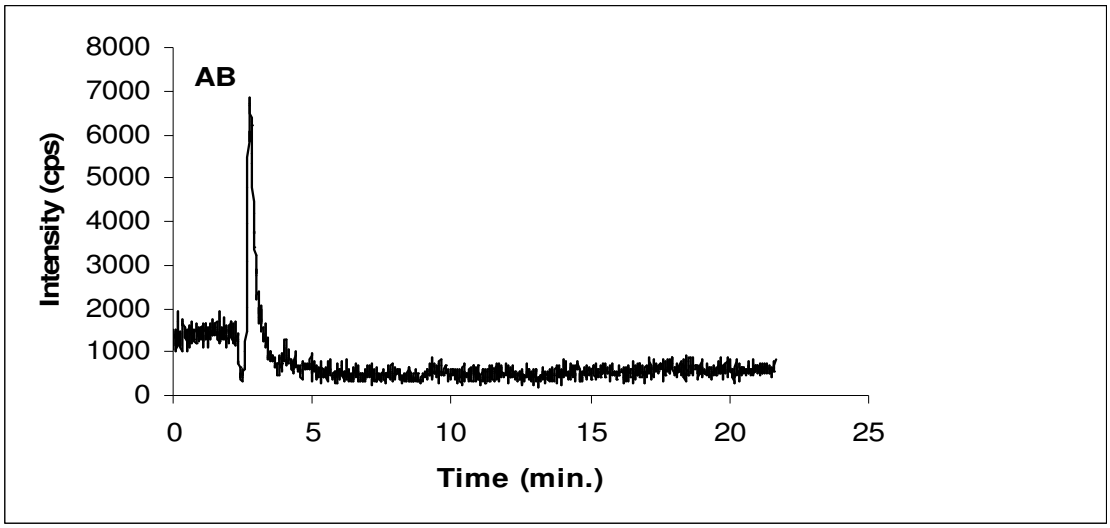

Figure 2.22 HPLC-ICP-MS chromatogram of a urine sample (UA1) 5-fold diluted with the mobile phase.

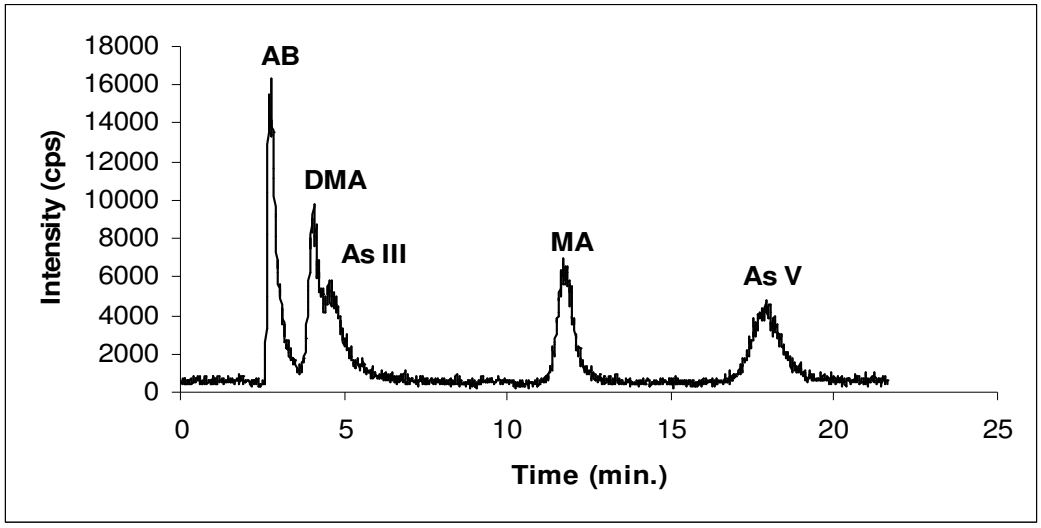

Figure 2.23 HPLC-ICP-MS chromatogram of a spiked urine sample (UA1) with 10 µg As/l of each arsenic species AB, DMA, As (III), MA and As (V).

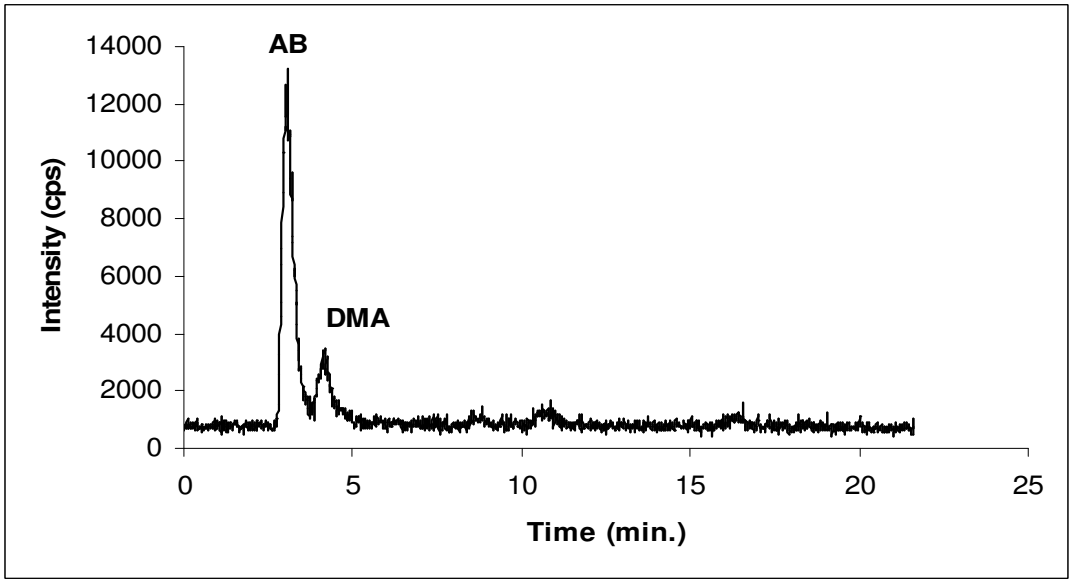

Figure 2.24 HPLC-ICP-MS chromatogram of a urine sample (UW18) 5-fold diluted with the mobile phase.

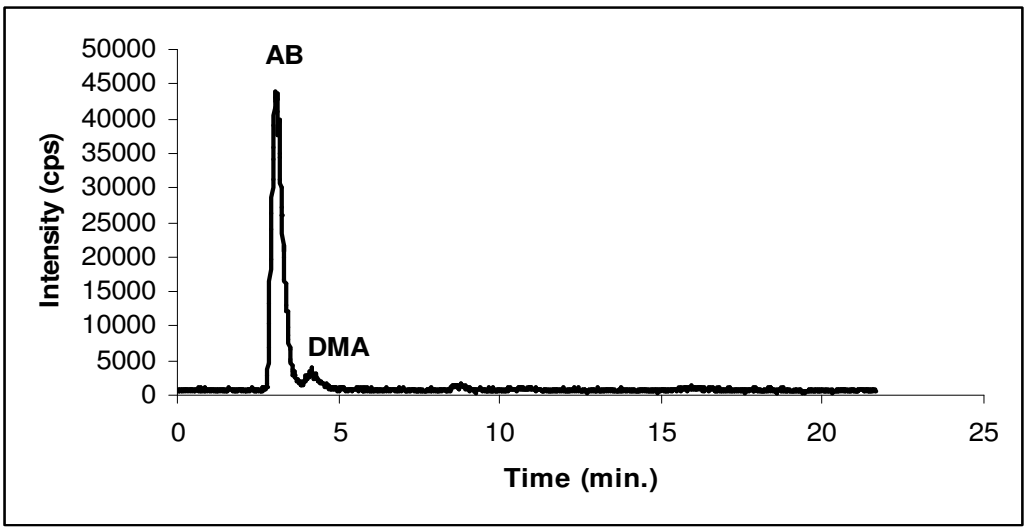

Figure 2.25 HPLC-ICP-MS chromatogram of a spiked urine sample (UW18) with 10 µg As/l of AB.

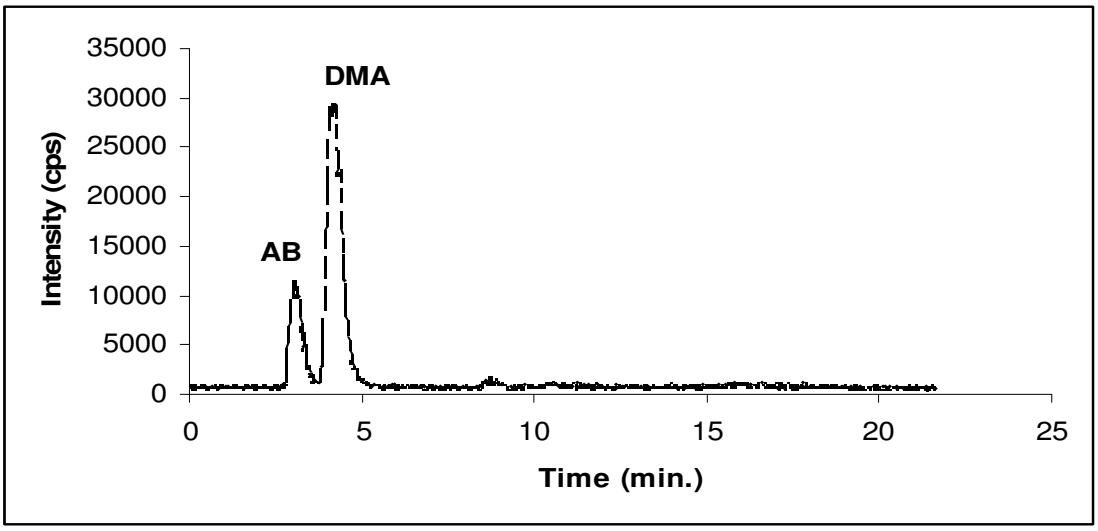

Figure 2.26 HPLC-ICP-MS chromatogram of a spiked urine sample (UW18) with 10 µg As/l of DMA.

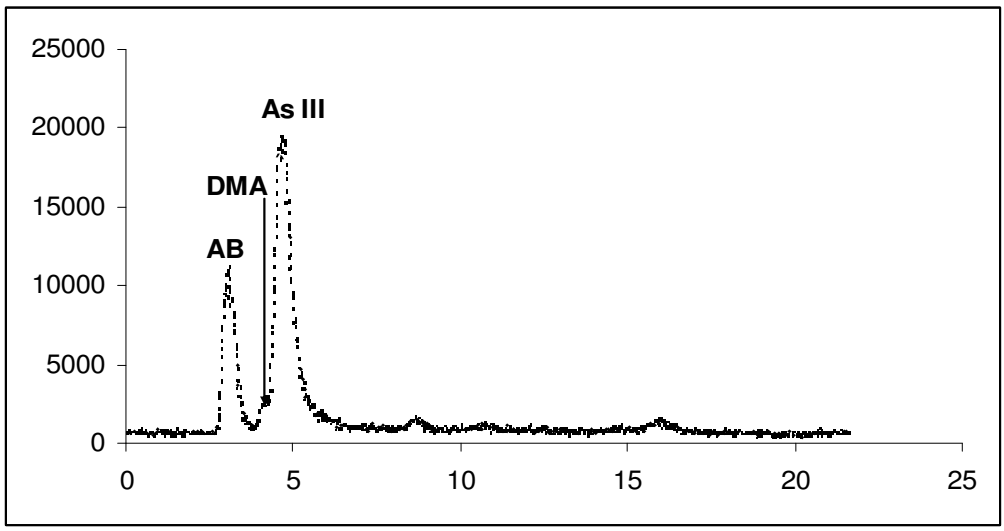

Figure 2.27 HPLC-ICP-MS chromatogram of a spiked urine sample (UW18) with 10 µg As/l of As (III).

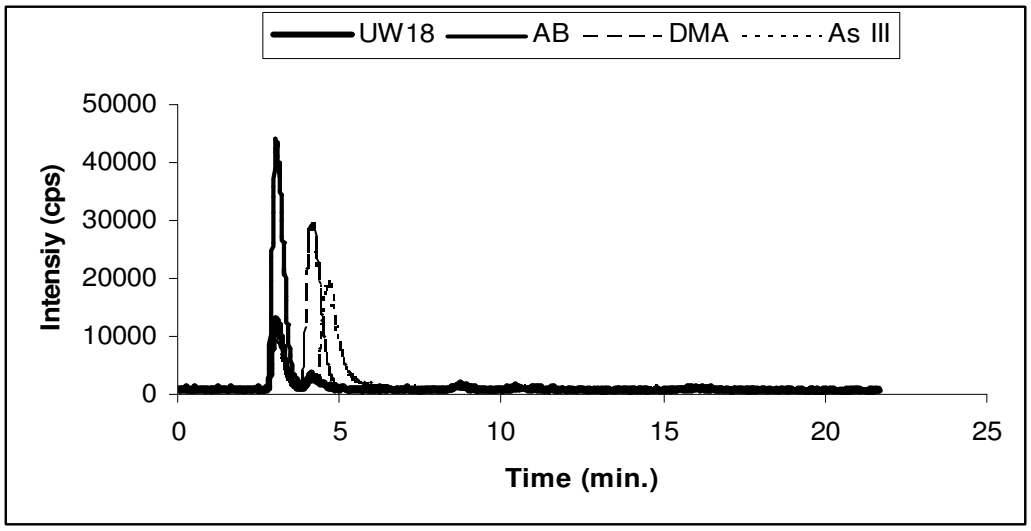

Figure 2.28 Overlaid chromatograms of urine sample UW18 (▬) spiked with AB 10 µg As/l (▬)and DMA 10 µg As/l (–)and As (III) 10 µg As/l (---). Chromatographic conditions are given in Table 2.20.

2.2.6 Statistical analysis

This section briefly describes the main statistical methods used in this thesis. More details on these methods can be found in such sources as Miller and Miller (2000) and Kerr et al., (2002). There are two types of statistical analysis used in most research investigations.

(i) **Descriptive statistics:** summarise the characteristics of the data set such as mean, median, standard deviation, variance and range.

(ii) **Inferential statistics:** are used to test a hypothesis and make inferences about sample data, which can be generalised to a larger population (Kerr et al., 2002).

In this research, Student's *t*-test and Kolmogorov-Smirnov's test (KS-test) were used for data analysis.

The collected data can be normally or not normally distributed. The former is distributed in the form of a bell-shape curve. The distribution is a mathematical concept and is defined by distance from the mean in standard deviation units; the same proportion of the area of the curve is always cut off. Therefore, the mean and the standard deviations provide a summary description of data that estimates the normal distribution (Kerr et al., 2002).

All the following definitions are reported by Kerr et al. (2002) in their book ''Doing statistics with SPSS''.

Normal distribution: A theoretical distribution that is symmetrical and in which the distribution of data is described by z-tables. For example, approximately 68% of the

population lies within ±1 standard deviations of the mean, and the equivalent figures for 95% and 99.7% are ±2 and ±3 respectively.

Depending on the type of distribution, either parametric or non-parametric statistical tests will be used to analyse the data. Parametric tests such as *t*–test are based on the assumption that data are drawn from a normally distributed population. By contrast, non-parametric tests such as KS-test make no assumption about data distribution. The former are more likely to detect significance differences between two sets of data, because they make more assumptions about those data (Kerr et al, 2002; Miller and Miller, 2000).

Non-parametric tests: Tests that make no assumptions about the distribution of the data.

Parametric tests: Tests which use the sample data to estimate the population parameters. They require at least interval data, equal variance and a normal distribution.

Some equations that show how different statistical measurements and tests can be derived are given in Miller and Miller (2000):

Mean: A measure of central tendency calculated by adding all the scores and dividing by the number of scores.

$$\bar{x} = \frac{\sum x_i}{n}$$

where $\bar{x}$ the mean and n is the number of measurements.

Standard deviation: The square root of the variance. It measures the dispersion of the scores around the mean in normal distribution.

$$s = \sqrt{\sum_i (x_i - \bar{x})^2 / (n-1)}$$

where s is the standard deviation on and n is the number of measurements.

$$RSD = 100\ s/\bar{x}$$

where RSD is the relative standard deviation.

A significance test is used to test the truth of null hypothesis (H_0). The term null implies that there are no differences between the values under the test, with exception of random variation differences. This means that if the H_0 is true, then *t*-test calculates the probability (P) that the observed difference between the values is the result of random error. The lower the P values indicating that the observed difference happens by chance, the less likely H_0 is true. Usually H_0 is rejected if the P of the difference taking place by chance is less than 1 in 20, which is 0.05 or 5%. In such case the difference is said to be significant at 5% level, in which is P = 0.05, this gives the probability to reject the true (H_0) (Miller and Miller, 2000). In case of comparing two sets of data to explore the difference, H_0 will be rejected if P value is small (< 0.05) (Tools for science, 2006).

The *t*-test is used in order to decide where the difference between two samples means, $\bar{x}_1$ and $\bar{x}_2$ is significant.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

s is calculated from :

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)}$$

The paired *t*-test tests whether there is a significant difference between the results obtained from the same subjects under different conditions

$$t = \bar{d}\sqrt{n} / s_d$$

where $\bar{d}$ and S_d are mean and standard deviation of d respectively, (d is the difference between paired values).

H_0 = zero correlation, used to test the significant correlation

$$t = \frac{|r|\sqrt{n-2}}{\sqrt{1-r^2}}$$

where r is the correlation coefficient and r^2 is the coefficient of determination.

In one case, an odd ratio is used in this research to measure how frequently the analyte was detected, by comparing two sets of data drawn twice from the same subjects under different conditions. An example to show how the probability can be calculated was reported by UCLA (2006) For example the probability of detection of the analyte is 0.X – thus

In this thesis odd ratio was used in one case to measure how frequently detected is the analyte, by comparing two sets of data that drawn twice from the same subjects under different condition. It has been reported (UCLA, 2006) and example to show how the probability can be calculated. For example, the probability of detection of the analyte is 0.X, thus

P = 0.X

The probability of absence is then

$$Q = 1-p = 0.Y$$

The odds of detection are defined as:

$$\text{Odds (detection)} = p/q = 0.X/0.Y = Z$$

Thus, the odds of detection are Z to one, and the odds of absence will be q/p.

where X,Y and Z are numbers.

The John Turkey test was used to identify which data points are outliers. This test defines the data as outliers if the data points are 1.5*IQR above the third or below the first quartile, where IQR is the interquartile range, the range between the third and first quartiles and is a measure of statistical dispersion (Tools for science, 2006).

In order to determine the statistical significance of the experimental data presented, the statistical methods described in this section have been applied where necessary to the experimental data in Chapters 3, 4 and 5.

2.3 Summary discussion of modified/developed methods

Here a brief discussion is presented to provide the rational behind the choice of analytical methods used in this project for the analysis of arsenic in biological samples.

Different spectroscopic techniques were used in this project: GF-AAS, ICP-MS and HPLC-ICP-MS. Suitable methodologies were modified and/or developed for preparation and analysis of arsenic in urine, hair and nail samples from human volunteers for analysis using spectroscopic techniques. ICP-MS and HPLC-ICP-MS were used for total arsenic and arsenic speciation analysis, respectively, especially for low levels of arsenic in human urine. These methods were found to be the most suitable techniques based on validation studies carried out.

In the development stage of the project the suitability of several spectroscopic techniques were evaluated for the analysis of arsenic in biological samples. The techniques used are those that were readily accessible for the purpose of this research project. The preparation and analysis of urine samples were carried out according to previous published procedure with appropriate modifications whenever necessary. Matrix effect is often a problem in the analysis of urine samples because it can either suppress or enhance the measured signal response of arsenic in urine. This problem was overcome for GF-AAS analysis by extensive dilution of the urine sample with the a diluent, in addition to the usage of a chemical modifier. The matrix effect due to chloride interference on arsenic in urine samples when using ICP-MS is considered as a disadvantage of this technique. This problem was overcome by using a correction equation, which eliminates the interference from chloride for determination of total arsenic in urine samples. While for arsenic speciation analysis by HPLC-ICP-MS in human urine, the problem of chloride interference was overcome through chromatographic separation. A successful elimination of the chloride was achieved through this method. This enabled a good separation and high recovery of arsenic species in the urine samples.

The above mentioned analytical methods were validated either *via* spiking experiments or with certified reference materials. High recoveries were achieved and the values determined with these methods were identical with the reference values given for either total arsenic or arsenic species. Consequently, these methods were used during the course of this project.

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Chapter 3

Ethnicity and Arsenic Metabolism

This Chapter investigates the role of ethnicity in understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of volunteers from three ethnic groups in Leicester, UK.

3.1 Introduction

Arsenic is a ubiquitous element and occurs in different chemical forms in the environment. In recent years, there has been much interest in this element due to the contamination of drinking water in some parts of the World. Millions of people in Bangladesh and India are exposed to high levels of arsenic from contaminated groundwater (Steinmaus et al., 2005; Suzuki, 2005; Tokunaga et al., 2005). Occupational exposure to arsenic can take place through mining, copper smelting and other activities (Loffredo et al., 2003). Natural exposure to arsenic can occur through consumption of seafood, which contains mainly organoarsenic compounds, or drinking water that is dominated by inorganic arsenic species (Yamaushi et al., 1992; Vahter, 1994; Tokunaga et al., 2005; Loffredo et al., 2003). Chronic exposure to inorganic arsenic through drinking water has been associated with hyperkeratosis, spotted melanosis, skin pigmentation and lung cancer (Yoshida et al., 2004). The toxicity of

arsenic in humans is dependent on its chemical form with both As(III) and As(V) being the most toxic, MA and DMA are less toxic, and AB, which is mainly found in seafood, being considered non-toxic (Ritsema et al., 1998; Vahter et al., 2000; Shraim et al., 2001). Thanks to recent studies, much progress has been made in the understanding of the metabolic pathway of inorganic arsenic in humans (Suzuki, 2005). Two alternating steps are involved in inorganic arsenic metabolism: reduction of As (V) to As (III), followed by oxidative methylation of As (III). After human exposure, a substantial fraction of As (V) is reduced to As (III) in the blood, which is then taken up by hepatocytes and methylated to MA and DMA (Hsueh et al., 1998). These latter methylated metabolites are less toxic than inorganic arsenic, and because of this, the methylation process is considered a detoxification mechanism (Hsueh et al., 1998; Adair et al., 2005). However, MA (III) and DMA (III), which have been identified in the urine of populations exposed to inorganic arsenic in their drinking water, have been shown to be more genotoxic than inorganic arsenic (Hsueh et al., 1998). Therefore, from a toxicity perspective, arsenic speciation in urine has an important role in understanding human health effects and the metabolism of arsenic. The half-life of inorganic arsenic in the body is 2 days, so measuring arsenic in urine can reflect an individual's recent exposure (Watanabe et al., 2001). However, reliance on only urine analysis may not provide a complete picture of arsenic metabolism when comparing the relationship between different factors, such as ethnicity and diet. It is known that arsenic levels in hair (Raab and Feldmann, 2005; Yanez et al., 2005; Mandal et al., 2003; Rahman et al., 2000), fingernail and toenail (Kile et al., 2005; Wilhem et al., 2005; Mandal et al., 2003) samples provide an additional level of information regarding arsenic exposure over a longer time scale. This is because sequestering of arsenic in

these tissues occurs over a longer time frame of 2–18 months (Tobin, 2005; Yoshinaga et al., 1990; Nowak and Kozlowski, 1998) compared to urine samples 3–4 days (Buchet et al., 1981). Progress in the understanding of arsenic metabolism in humans is hindered by the fact that little is known about arsenic metabolism in healthy populations. In recent years, several studies have reported on this subject with respect to arsenic metabolism in populations exposed to high levels of arsenic through consumption of contaminated drinking water (Hopenhayn-Rich et al., 1996; Mandal et al., 2003; 2004; Kile et al., 2005).

The awareness of the relationship between ethnicity, diet and arsenic metabolism is rather poor, particularly for populations exposed to low concentrations of arsenic. Ethnicity is defined as large group of people with a common background, such as culture, religion, language, nation, tribe or race (Merriam-Webster's online dictionary, 2006). Studies conducted on this subject suggest that dietary (Lai et al., 2004) and ethnic differences (Loffredo et al., 2003; Hulle et al., 2004) may play a role in arsenic metabolism and there are also implications for the role of genetics (Hopenhayn-Rich et al., 1996; Vahter et al., 2000; Loffredo et al., 2003; Kile et al., 2005) in arsenic metabolism. However, these studies have focused on populations exposed to high levels of arsenic and often involved analysis of just urine samples.

Keeping these two limitations in mind, this is the first large study to investigate the role of ethnicity in arsenic metabolism in unexposed populations from the UK. This was carried out by analysis of hair, fingernails and urine samples of volunteers, from three different ethnic communities, residing in the same city (Leicester, United Kingdom), who have not been exposed to high levels of inorganic arsenic through drinking water or environmental and occupationional exposure.

3.2 Experimental

This section covers study group, sample collection, stability of arsenic species, material and methods, and statistical analysis. All of these sub-sections also apply to all urine samples discussed in Chapter 4 and 5, unless otherwise stated.

3.2.1 Study group

Healthy volunteers from White, Asian and Somali Black-African communities were recruited from Leicester, UK. The total number of participants for the urine analysis was 63 adults [mean age 31.8 years (4 volunteers did not report their age); 21 women, 42 men]. For the hair and fingernails analysis, 36 volunteers were recruited. These were mainly new volunteers although some of them also provided urine samples, which were included in the urine analysis data. The mean age of the hair and nail analysis volunteers was 34.6 years [(four volunteers did not report their age); 11 women, 25 men]. In order to ensure that the volunteers were not exposed to arsenic in their drinking water, GF-AAS was used to determine the arsenic content of drinking water, from the water supply system, in Leicester. The analysis revealed that the level is below the detection limit of the technique (0.25 μg/l).

Each individual was asked to refrain from eating fish and seafood only for 3 days prior to sample collection while other factors such as alcohol, smoking etc were recorded using a questionnaire. The volunteers were asked to complete a questionnaire asking for demographic information such as ethnicity, age, gender, and life-style characteristics such as diet, smoking and alcohol consumption. Based on the responses

to the questionnaire, the participants were classified into three ethnic groups: Asian (Appendix 3.1), Somali Black-African (Appendix 3.2) and White (Appendix 3.3). Appendices 3.1, 3.2 and 3.3 provide detailed information (demographic factors and life style) about these three ethnic groups. The Asian group comprised individuals with ancestral links to India, Pakistan, and Bangladesh. The Somali Black-African population was made up of individuals originating from Somalia, East Africa, and the White group comprised indigenous white people from the UK. All of the subjects were resident in Leicester, UK at the time of the study. The Faculty of Health and Life Sciences Human Research Ethics Committee, at De Montfort University, Leicester, approved the study's design and aims.

3.2.1.1 Time delay between urine samples collection, storage and analysis

During the course of this project, there were time delays between sample collection storage and measurement. This is not unusual in the analysis of urine samples using analytical techniques especially those studies involving large number of samples from diverse groups of people. It is important to point out that conditions that have been used in this study such as sample collection, storage and measurements procedures are very similar to what has been used by others in the analysis of arsenic species in human urine samples. Presented below is a discussion, which shows that the arsenic species investigated in this project are stable for many months.

The urine samples used in the project were transported at room temperature by the volunteers to the laboratory. There is also some time delay between collection and storage of urine samples, which can arise from variation in the time (some hours) of

collection by the volunteers. Such transportation and time delays associated with analysis of urine samples is a common practice in the analysis of arsenic in human urine (Heitland and Koster 2006; Morton and Mason 2006). There is literature evidence for stability of arsenic species at room temperature. For example, based on previous studies (Feldman et al., 1999; Palacios et al. 1997) on stability of arsenic species and storage conditions, the following species MA, DMA and AB in urine sample were stable for 67 days at ambient temperature. In addition As (III), As (V), MA and DMA were less stable at 25 ^{0}C for up to 2 months , while AB was stable up to 8 months at 25 ^{0}C. Therefore, transporting urine samples at room temperature was considered to have no effect on the stability of these species.

Urine samples used in this thesis were stored at -20 ^{0}C before analysis. Storage of urine samples at -20 ^{0}C for many months prior to analysis is common (Aposhian et al., 1997). The longevity in their study was 6 months, and then the urine samples were thawed at room temperature overnight prior to the measurement. Few studies have been reported in the literature regarding stability of naturally occurring arsenic species in human urine some of which are discussed below.

Naturally occurring species, such as DMA and MA, in human urine samples has been reported to be stable for 8 months when stored at -20 ^{0}C (Feldmann et al., 1999). The samples were measured twice: 2 weeks and 8 months after the sample collection. The concentration of AB added to urine sample from a volunteer, has been reported (Feldmann et al., 1999) to remain unchanged for up to 8 months under storage condition of -20 ^{0}C; with measurements being carried out three times (2, 4 and 8 months) after sample collection.

Chen et al. (2002) have reported stability of five naturally occurring arsenic species (AB, DMA, MA, As (III) and As (V)) in seven freshly collected human urine samples, and also in a certified urine sample (NIST SRM 2670). These species were found to be stable for up to six months when the samples were stored at -20 ^{0}C. Arsenic levels in these samples were measured weekly over the period of the study. Other authors (Cornelis et al., 1995) have also reported that arsenic in human urine is stable over 6 months when stored at -20 ^{0}C.

A stability study for naturally occurring arsenic species such AB and DMA in freeze-dried urine (CRM NIES No.18) over a period of 2 years has been carried out by Yoshinaga et al. (2000). The stability of DMA and AB was investigated for one and 2 years respectively, by analysing freshly reconstituted samples. The concentrations of these species were found to be stable under storage condition for the freeze-dried CRM at 4 ^{0}C.

In this study AB, DMA and MA were the main species analysed and, as discussed above, these arsenic species are highly stable for several months. Procedures used in this study are analogous to what has been used by other researchers in the field of arsenic analysis in human urine.

3.2.1.2 Urine samples collection, storage and preparation

Urine samples collection and storage were carried out as generally reported in the literature (Steinmaus et al., 2005; Hopenhayn-Rich et al., 1996; Vahter et al., 1995). First morning urine samples were collected in polyethylene bottles from the three healthy ethnic groups (White n = 20; Asian n = 21; and Somali Black-African n = 22), residing in Leicester, UK. Volunteers were asked to refrain from eating fish and

seafood for 3 days prior to sample collection, and to complete a questionnaire, which gathered information on age, gender and ethnicity along with lifestyle. The questionnaire was accompanied with a letter to explain, to each of the volunteer, the details of the project and how to deal with the samples in terms of collection and storage. Morning urine samples (mid-stream) were collected directly into polyethylene bottles (Fisher, UK). Once in the laboratory the normality of the urine sample was established by using a Combur-9 urine test strip (Roche, Germany). The samples were then stored at – 20 °C until the analyses were carried out. Prior to total arsenic or arsenic speciation analysis, the samples were filtered through a 0.45 μm pore syringe filter and diluted up to 5-fold with the mobile phase for speciation analysis, and 5-fold with 2%v/v HNO_3 for total arsenic determination.

All urine samples were transported at normal temperature to the laboratory (frozen upon receipt) or to the location of the measurement, and the time delay between storage (– 20 °C) and measurement was in the range of 1 – 6 months (Appendices 3.4, 3.5 and 3.6).

3.2.1.3 Dates of collection and measurements of urine samples from the three ethnic groups

Here, dates of collection and dates of measurements and locations for arsenic analysis of all urine samples from the three ethnic groups (Asian, Somali and Whites) are described in detail. Speciation measurements of arsenic in the urine samples from all ethnic groups (Asian, Somali and whites in Appendices 3.4, 3.5 and 3.6, respectively) were carried out at The University of Manchester (HPLC-ICP-MS, PQ II, VG Instruments, Winford, UK). The total arsenic in the urine samples from the three

ethnic groups (Appendices 3.4, 3.5 and 3.6) were measured at The University of Hull (ICP-MS, ELAN DRCII, PerkinElmer SCIEX, Concord, Ontario, Canada).

3.2.2 Chemicals and reagents

Deionised water (>18Ω cm−1) was used throughout the study. Stock solutions of arsenic species were prepared and standardised against an As (V) standard (1000 ± 3 mg/l, CPI, International, USA). As (III) [(As_2O_3), Sigma-Aldrich,Germany] was dissolved in 4g/l sodium hydroxide and made up to appropriate volume with 2% v/v HNO_3 (UPA, Romil, UK). Stock solutions of As (III), As (V), DMA [$(CH_3)_2AsO(OH)$, Sigma-Aldrich, Germany], MA [$CH_3AsO(OH)_2$, Greyhound, Dorset, England] and AB [$(C_5H_{11}AsO_2$, Fluka, Fisher Chemicals, UK] were prepared in deionised water and stored in the fridge at 4 °C. Fresh diluted solutions were prepared daily for analysis. The mobile phase (20 mM NH_4HCO_3, pH 10.3) was used for arsenic speciation, which was mentioned before in Section 2.2.3 (Table 2.6).

3.2.3 Instrumentation

Methods have been developed for both total arsenic and arsenic speciation analysis. Details regarding this can be found in Chapter 2 (Sections 2.2.4 and 2.2.5, respectively).

3.2.4 The procedure to determine the effect of seafood consumption on the background level of total urinary arsenic

Different seafood consumed by one volunteer, and the effect of these seafood on the background of total urinary arsenic level was evaluated. The following quantities were ingested from the different foods: salmon (350 g), prawn (300 g), seaweed (15 g) and cod liver oil (10 ml). First morning void urine samples were collected from the volunteer on the day before ingestion time (0 day) and then every day up to seven days after the ingestion. The results are presented in section 3.3.1.1.

3.2.5 Quality control and method validation

CRM or known standard were also analysed during each analytical run as a quality control check. The method validation for total arsenic and arsenic speciation analysis was carried out and is same as what has been reported in Chapter 2: sections 2.2.4 and 2.2.5, respectively. The validation of total arsenic in hair was mentioned in section 2.2.2.7.

3.2.6 Determination of creatinine

The creatinine measurement for this Chapter was the same to what has been reported in Chapter 2 (section 2.2.1.3).

3.2.7 The procedure to determine the effect of storage time on arsenic compound stability

An experiment was carried out to evaluate the stability of naturally occurring arsenic species in human urine, which was freshly collected from a volunteer. A urine sample was collected from the volunteer who had refrained form eating fish and seafood for 3 days prior to sample collection. The sample was stored at -20^{o}C and then subjected to total arsenic measurement over a period of sixteen months: results are shown in section 3.3.1.2. An other experiment was also carried out for arsenic speciation analysis over a period of six months: results are shown in section 3.3.1.3.

3.2.8 Determination of total arsenic in urine samples

All urine samples were filtered through a 0.45 μm pore syringe filter and diluted 5-fold with 2%v/v HNO_3 for total arsenic determination. The calibration curve of arsenic standard is shown in Chapter 2 (see Fig. 2.13). The determination of total arsenic in urine samples was carried out same as described in Chapter 2 (see section 2.2.4).

3.2.9 Speciation analysis of arsenic in urine samples

In order to see if there were any differences in arsenic speciation in the urine samples from the three ethnic groups, HPLC separation of AB, MA, As (III), As (V) and DMA was carried out. The calibration curves are shown in Chapter 2, section 2.2.5, Fig 2.15. The problem of interference from high levels of chloride present in the urine samples was overcome as is shown in Chapter 2, section 2.2.5, Fig. 2.16. The chloride was chromatographically resolved from the various arsenic species.

3.2.10 Collection, pre-treatment and total arsenic determination of hair and fingernail samples

Hair samples from different positions of the head and fingernails, from all 10 fingers, were collected from the three ethnic groups (White n = 11, Asian n = 10 and Somali Black-Africans n = 15). All samples were kept in sealed plastic bags and stored at room temperature. The hair and fingernails samples were washed and analysed as described before in Chapter 2, section 2.2.2.7.

3. 2.11 Statistical analysis

Differences between ethnic groups in terms of arsenic species in urine and total arsenic levels in urine, hair and fingernail were evaluated using the *t*-test. The difference is significant when $P > 0.05$ at 95% confidence interval. The association of age and gender were also evaluated by using the same test.

3. 3 Results

All results regarding urine samples (total arsenic and arsenic species), hair and fingernails (total arsenic) are presented in the following sections:

3. 3. 1 Analysis of urine samples

This section includes the stability experiment on urine samples for storage and the effect of seafood's consumption on the background level of urinary arsenic.

3.3.1.1 Effect of seafood consumption on the background of urinary arsenic level

The main objective of this experiment was to estimate the contribution of seafood ingestion on the background level after one day from ingestion time. This experiment was also used to confirm the literature reports (Hsueh et al., 2002; Palacios et al., 1997) that arsenic from seafood is cleared after 3 days. The individual data is presented in Table 3.1 and graphically illustrated in Fig 3.1. It is apparent that three days is sufficient for clearance of arsenic from human body through urine, especially arsenic that originates from seafood and seaweed. After three days of seafood and seaweed ingestion, the measured level was almost the same as the background level before this type of food was consumed.

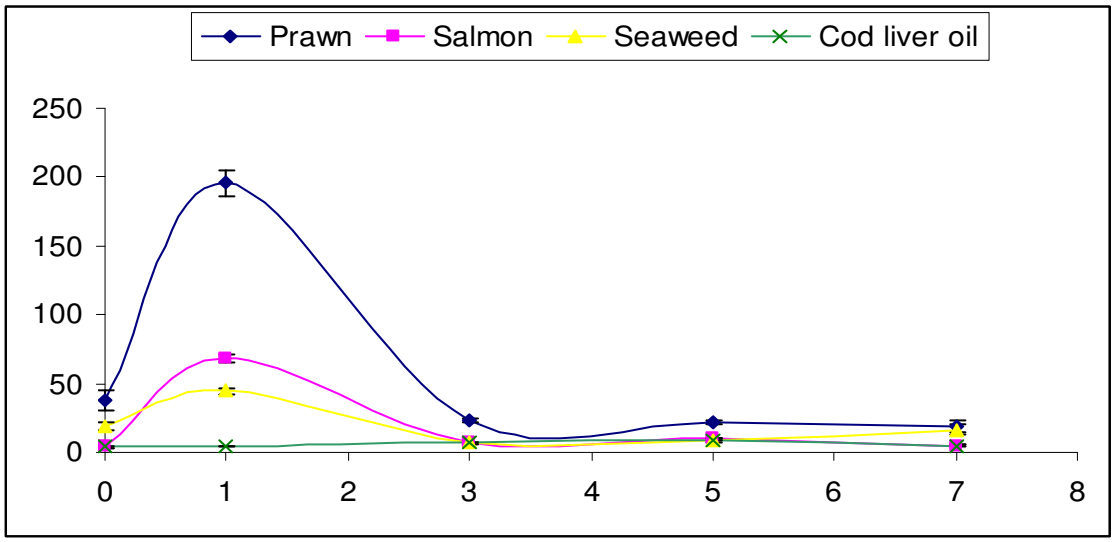

Figure 3.1 The effect of seafood ingestion on background level of urinary total arsenic level; data points are means; bars are SD (n = 3).

Table 3.1 Total arsenic (µg/l) and creatinine (mg/l) in urine samples collected from one volunteer after seafood ingestion. All urine samples were measured on 17/08/2005, at The University of Hull by ICP-MS.

Ingested food, dates of ingestion	Days after food ingestion	Urinary total arsenic conc. (µg/l)	Creatinine (mg/l)	Urinary total arsenic conc. (µg As/g creatinine)	SD (n=3)
Salmon, 30/05/2005	0	9.9	2398.0	4.1	0.7
	1	143.8	2115.0	68.0	3.1
	3	14.7	2149.0	6.9	0.3
	5	16.1	1561.0	10.3	0.4
	7	8.5	1810.0	4.7	0.8
Cod liver oil, 27/06/2005	0	10.2	2613.0	3.9	0.7
	1	13.4	2726.0	4.9	0.2
	3	9.5	1425.0	6.6	0.2
	5	14.4	1776.0	8.1	0.3
	7	15.2	3110.0	4.9	0.9
Seaweed, 07/07/2005	0	11.6	611.0	18.9	3.2
	1	120.6	2703.0	44.6	2.2
	3	19.6	2647.0	7.4	0.3
	5	26.5	2873.0	9.2	0.4
	7	30.9	1855.0	16.7	3.2
Prawn, 27/07/2005	0	65.9	1753.0	37.6	7.6
	1	392.8	2002.0	196.2	9.4
	3	66.2	2816.0	23.5	1.1
	5	46.5	2138.0	21.7	1.0
	7	47.3	2500.0	18.9	3.7

3.3.1.2 Relationship between storage conditions and the stability of total arsenic in urine samples

A urine sample was collected on 01/10/2004 from a volunteer with no recent seafood ingestion, spiked with 200 µg/l of As(V) on 04/04/2004 (100404QC). The sample was then stored at -20 ^{0}C. Subsequently analysis for total arsenic was carried out using GF-AAS. The sample was diluted 200-fold with 1%v/v HNO_3 prior to analysis. Table 3.2 shows the dates of the analysis and measured concentrations in the diluted sample, and total arsenic concentrations were obtained by multiplying the measured concentration with the dilution factor. A series of analysis were carried out over 16 months, with an average 243.7 µg/l of total arsenic and very low standard deviation (6.4) and 2.6%RSD (n = 6). This shows the stability of total arsenic in urine over a period of approximately one and a half years .

Table 3.2 Measured concentration (µg/l) of arsenic in diluted (200-fold) urine sample (100404QC) and dates of analysis**.**

Dates of analysis	Measured arsenic conc. (µg/l)	Abs.		Total arsenic conc. (µg/l)	SD (n = 3)
28/10/2004	1.22	0.0114		244	0.60
01/12/2004	1.25	0.0087		250	0.25
04/02/2005	1.28	0.0112		246	0.17
17/05/2005	1.16	0.0064		232	0.34
29/07/2005	1.21	0.0097		242	0.11
28/02/2006	1.24	0.0074		248	0.38
Mean	**1.23**			**243.7**	
SD	**0.04**			**6.4**	
%RSD	**3.3**			**2.6**	

3.3.1.3 Relationship between storage conditions and the stability of arsenic species in urine samples

The results of the experiment on the effect of urine storage condition on arsenic compounds stability showed that the arsenic species (AB and DMA) remained constant during the course of the study. The chromatograms in Fig. 3.2 and the determined values are as follows: AB one month after collection (19.2 ± 1.5µg As/l) and 6 months after collection (19.3 ± 0.2 µg As/l); DMA one month after collection (4.0 ± 0.8µg As/l) and 6 months after collection (4.3± 0.2 µg As/l), at -20 ^{0}C storage condition. The average between of the two measurements were 19.3 ± 0.1 µg As/l and 4.2± 0.2 µg As/l for AB and DMA, respectively. These results are in agreement with reports of the literature, that these arsenic species are highly stable in human urine under similar storage condition. Therefore,the relevant arsenic species are stable over the period of the study.

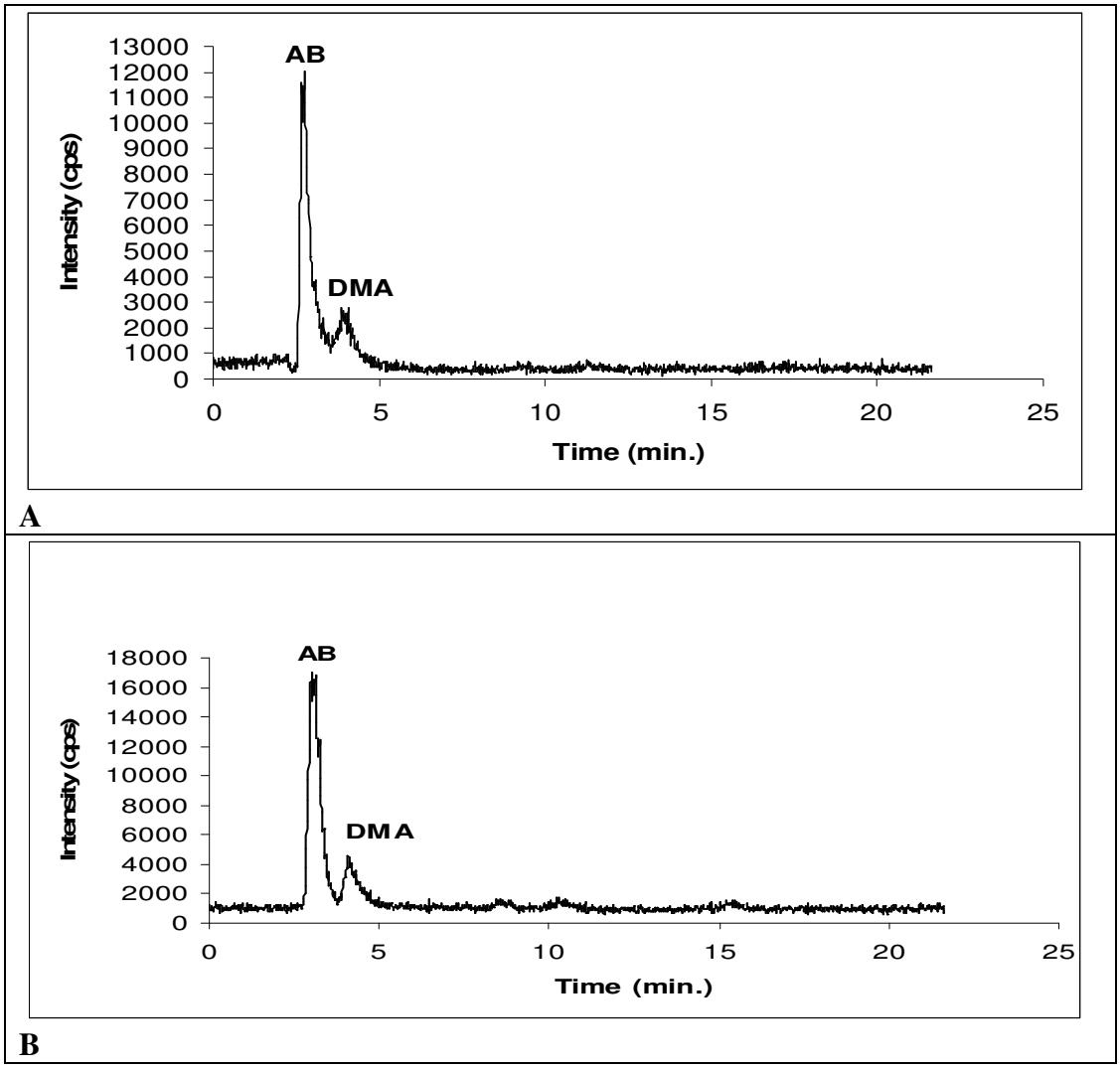

Figure 3.2 Chromatograms obtained from the HPLC-ICP-MS analyses of two arsenic species in urine sample (UW18) within 1 month; AB (19.2 ± 1.5µg As/l) and DMA (4.0 ± 0.8µg As/l) (A) and 6 months; AB (19.3 ± 0.2 µg As/l) and DMA (4.3± 0.2 µg As/l) (B) of collection and storage at -20 ^{0}C.

3.3.1.4 Representative chromatograms for speciation of arsenic in urine samples

Representative chromatograms for speciation of arsenic in urine samples from three volunteers from Asian, Somali and White ethnic groups are presented in Figures

3.3, 3.4 and 3.5, respectively. Additional representative figures are presented in Appendix 3.7, which includes chromatograms from blank, standard solutions, certified urine sample (CRM NIES No.18) and real samples. The results of the CRM NIES No. 18 were as follows: AB 63.3 ± 0.9µg As/l and DMA 38.0 ± 1.2 µg As/l; the certified values were 69 ± 12 µg/l and 36 ± 9µg/l, respectively. The reproducibility of the method was also validated by measuring 10 µg As/l standard mixture of the five arsenic species after each 6 runs. The reproducibility (n =3) was 9.7 ± 0.2µg As/l (2.4% RSD) for AB, 10.3 ± 0.6 µg As/l (6.2% RSD) for DMA, 10.0 ± 0.3 µg/l (3.3% RSD) for As (III), 10.5 ± 0.2µg As/l (1.7% RSD) for MA and 10.2 ± 0.3 µg/l (2.8% RSD) for As (V).

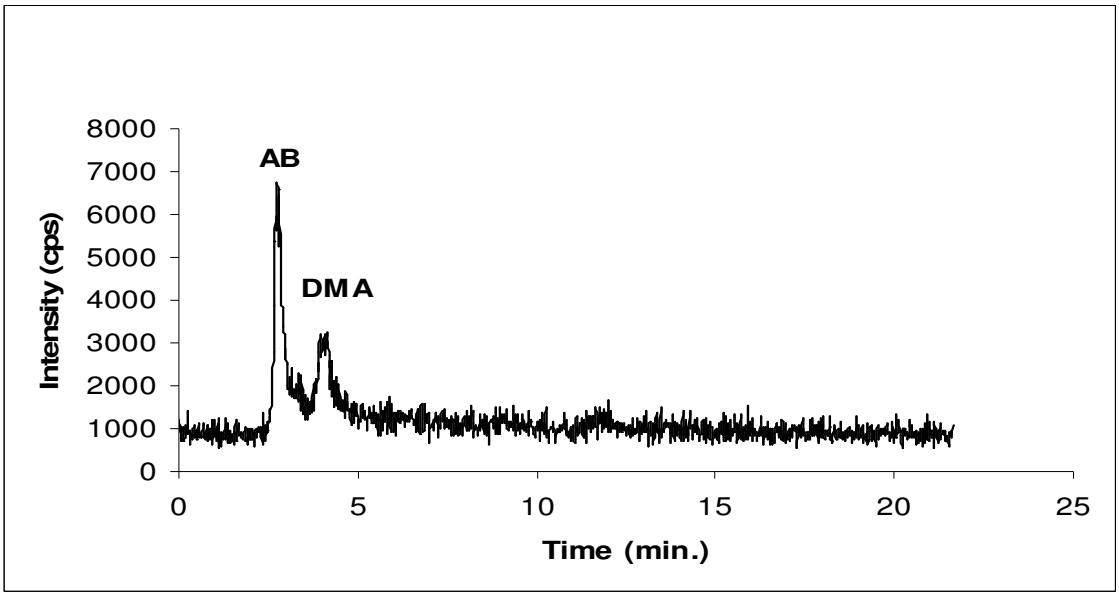

Figure 3.3 Example of a HPLC-ICP-MS chromatogram for speciation of arsenic in the urine of an Asian volunteer (UA21). Species concentrations are AB (12.5 ± 0.4 µg As/l) and DMA (5.4± 0.4 µg As/l)

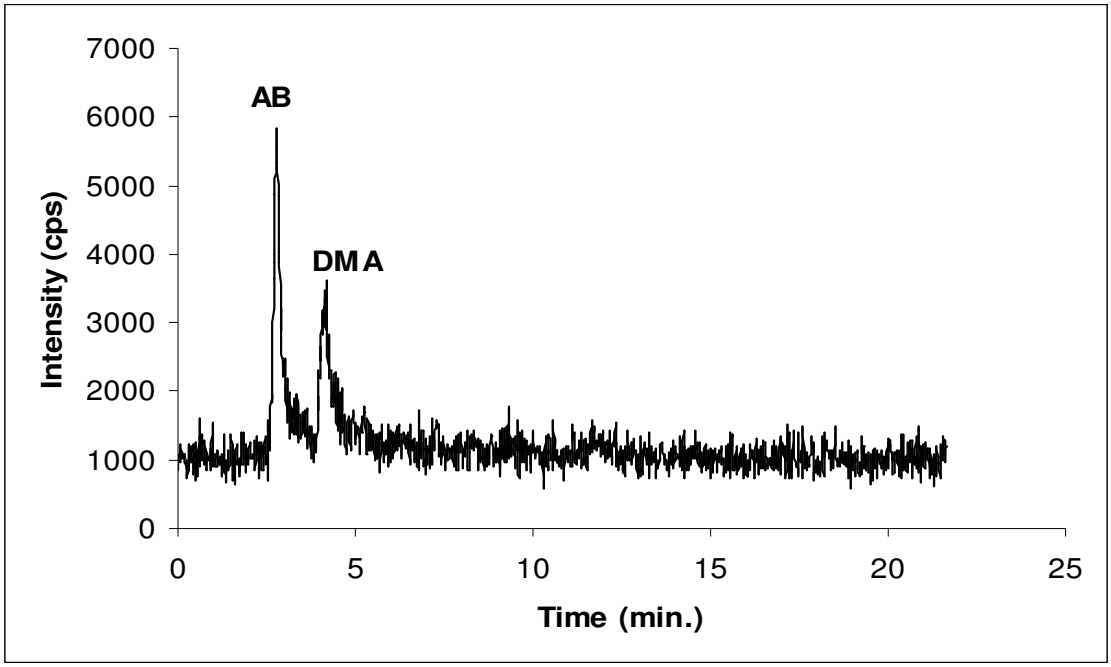

Figure 3.4 Example of a HPLC-ICP-MS chromatogram for speciation of arsenic in the urine of a Somali volunteer (US5). Species concentrations are AB (8.4 ± 0.2 µg As/l) and DMA (5.3± 0.3 µg As/l)

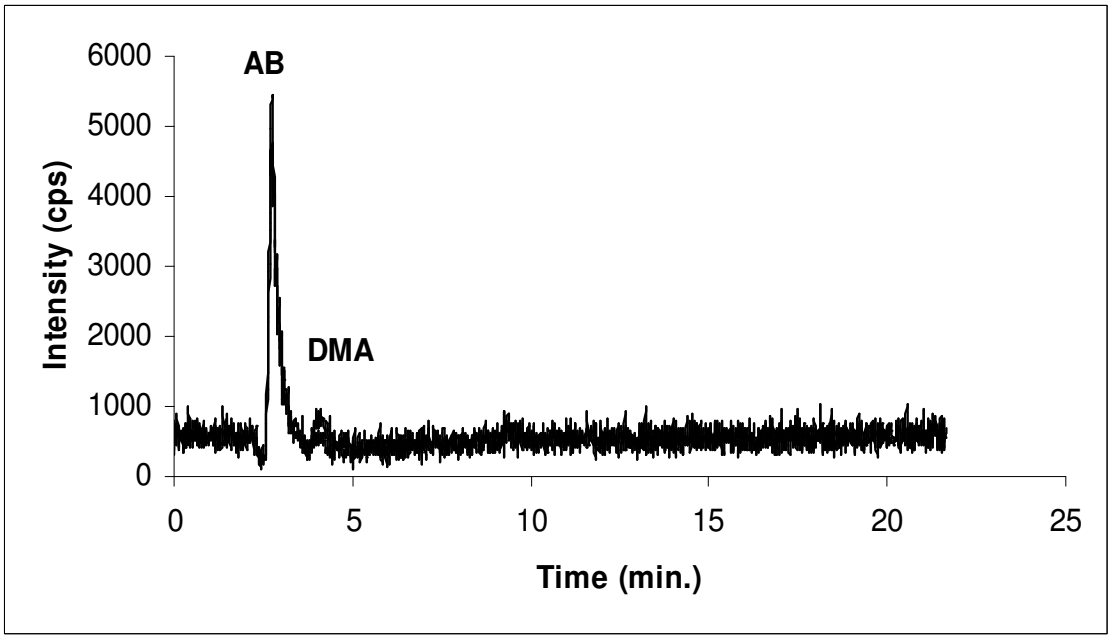

Figure 3.5 Example of a HPLC-ICP-MS chromatogram for speciation of arsenic in the urine of a White volunteer (UW12). Species concentrations are AB (18.7 ± 0.4 µg As/l) and DMA (1.6± 0.6 µg As/l)

3.3.1.5 Concentration of total arsenic and arsenic species before creatinine adjustment among the three ethnic groups

This section shows total arsenic concentration (µg/l) and arsenic species (AB, DMA and MA) concentrations (µg As/l), before creatinine adjustment, among the three ethnic groups: Asian, Somali and White in Table 3.2, 3.3 and 3.4, respectively.

Table 3.3 Asian group: Concentration of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) urine samples of the Asian group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic	Creatinine (mg/l)
1.	UA1	26.5	<LOD	<LOD	26.5	30.5	1868
2.	UA2	3.0	1.9	<LOD	4.9	17.9	3136
3.	UA3	50.5	8	<LOD	58.5	70.5	1709
4.	UA4	43.5	2.5	<LOD	46.0	55.2	1823
5.	UA5	81	11.5	<LOD	92.5	60.4	2139
6.	UA6	15.5	14.1	<LOD	29.6	47.3	2151
7.	UA7	195.0	30.5	<LOD	225.5	198.8	2355
8.	UA8	12.0	1.0	<LOD	13.0	17.4	1347
9.	UA9	<LOD	3.7	<LOD	3.7	11.8	1166
10.	UA10	7.0	1.1	<LOD	8.1	15.6	2332
11.	UA11	18.9	8.1	<LOD	27.0	40.7	1902
12.	UA12	29.5	6.4	<LOD	35.9	44.5	2264
13.	UA13	19.5	1.5	<LOD	21.0	25.2	1641
14.	UA14	12.2	1.6	<LOD	13.8	21.9	1562
15.	UA15	10.4	2.2	<LOD	12.6	18.6	2660
16.	UA16	3.0	1.4	<LOD	4.4	8.6	1075
17.	UA17	7.8	3.1	<LOD	10.9	16.5	1404
18.	UA18	95.6	7.2	1.8	104.6	63.8	1958
19.	UA19	<LOD	1.1	<LOD	1.1	6.5	283
20.	UA20	3.5	3.6	<LOD	7.1	10.1	1404
21.	UA21	12.5	5.4	<LOD	17.9	33.8	2434
	Mean	**30.8**	**5.5**	**0.1**	**36.4**	**38.8**	**1838.7**
	SD	**45.5**	**6.8**	**0.4**	**51.6**	**41.5**	**625.2**
	Median	**12.5**	**3.1**	**<LOD**	**17.9**	**25.2**	**1868.0**
	Min.	**<LOD**	**<LOD**	**<LOD**	**1.2**	**6.5**	**283.0**
	Max.	**195.0**	**30.5**	**1.8**	**225.5**	**198.8**	**3136.0**

Table 3.4 Somali group: Concentration of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) urine samples of the Somali group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic	Creatinine (mg/l)
1.	US1	1.2	1.0	<LOD	2.2	8.1	1030
2.	US2	<LOD	0.7	<LOD	0.7	4.6	758
3.	US3	<LOD	0.7	<LOD	0.7	3.1	826
4.	US4	1.7	3.2	<LOD	4.9	12.2	1664
5.	US5	8.4	5.3	<LOD	13.7	22.6	1811
6.	US6	2.4	1.0	<LOD	3.4	10.2	838
7.	US7	0.9	0.9	<LOD	1.8	7.5	1087
8.	US8	1.1	2.6	<LOD	3.7	13.2	1290
9.	US9	5.6	1.3	<LOD	6.9	10.7	724
10.	US10	<LOD	<LOD	<LOD	<LOD	4.5	634
11.	US11	<LOD	2.9	<LOD	2.9	7.7	577
12.	US12	<LOD	<LOD	<LOD	<LOD	3.2	634
13.	US13	<LOD	<LOD	<LOD	<LOD	3.4	577
14.	US14	<LOD	1.4	<LOD.	1.4	3.3	838
15.	US15	<LOD	4.0	1.0	5.0	6.2	1087
16.	US16	1.4	2.0	<LOD	3.4	7.8	906
17.	US17	2.5	1.3	<LOD	3.8	8.8	1449
18.	US18	<LOD	<LOD	<LOD	2.3	7.8	3804
19.	US19	<LOD	0.9	<LOD	0.9	2.0	5060
20.	US20	2.1	1.8	<LOD	3.9	12.5	2422
21.	US21	<LOD	<LOD	<LOD	<LOD	4.6	1336
22.	US22	7.5	0.7	<LOD	8.2	18.6	1743
	Mean	**1.6**	**1.4**	**<LOD**	**3.2**	**8.3**	**1413.4**
	SD	**2.5**	**1.4**	**0.2**	**3.3**	**5.2**	**1102.5**
	Median	**0.5**	**<LOD**	**<LOD**	**2.6**	**7.8**	**1058.5**
	Min.	**<LOD**	**<LOD**	**<LOD**	**<LOD**	**2.0**	**577**
	Max.	**8.4**	**5.3**	**1.0**	**13.7**	**22.6**	**5060**

Table 3.5 White group: Concentration of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) urine samples of the White group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic	Creatinine (mg/l)
1.	UW1	1.0	3.6	<LOD	4.6	28.6	1472
2.	UW2	1.0	2.4	<LOD	3.4	9.4	374
3.	UW3	<LOD	<LOD	<LOD	0.0	5.2	464
4.	UW4	75.3	<LOD	<LOD	75.3	56.7	1698
5.	UW5	9.3	5.2	<LOD	14.5	30.1	2332
6.	UW6	59.7	9.9	<LOD	69.6	61.7	940
7.	UW7	<LOD	4.0	<LOD	4.0	16.2	770
8.	UW8	2.4	1.0	1.6	5.0	9.5	996
9.	UW9	<LOD	2.1	<LOD	2.1	5.7	351
10.	UW10	2.8	5.9	4.1	12.8	23.3	1324
11.	UW11	2.1	4.4	<LOD	6.5	11.9	679
12.	UW12	18.7	1.6	<LOD	20.3	27.1	543
13.	UW13	21.8	<LOD	<LOD	21.8	24.2	340
14.	UW14	<LOD	4.7	<LOD	4.7	7.9	917
15.	UW15	1.5	1.9	<LOD	3.4	5.4	159
16.	UW16	2.2	2.7	<LOD	4.9	8.3	1053
17.	UW17	2.2	5.4	<LOD	7.6	10.1	951
18.	UW18	19.2	4.0	<LOD	23.2	17.9	419
19.	UW19	2.3	3.3	<LOD	5.6	9.1	1426
20.	UW20	<LOD	1.2	<LOD	1.2	4.2	475
	Mean	**11.1**	**3.2**	**0.3**	**14.5**	**18.6**	**884.2**
	SD	**20.6**	**2.4**	**1.0**	**21**	**16.2**	**549.6**
	Median	**2.2**	**3.0**	**<LOD**	**5.3**	**11.0**	**843.5**
	Min.	**<LOD**	**<LOD**	**<LOD**	**<LOD**	**4.2**	**159**
	Max.	**75.3**	**9.9**	**4.1**	**75.3**	**61.7**	**2332**

3.3.1.6 Concentration of total arsenic and arsenic species after creatinine adjustment among the three ethnic groups.

This section shows total arsenic concentration (µg As/g creatinine) and arsenic species (AB, DMA and MA) concentrations (µg As/g creatinine), after creatinine adjustment, among the three ethnic groups: Asian, Somali and White in Table 3.6, 3.7 and 3.8, respectively.

Table 3.6 Asian group: Concentration of total arsenic and arsenic species (µg As/g creatinine) in urine samples of Asian group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic
1.	UA1	14.2	0.0	0.0	14.2	16.3
2.	UA2	1.0	0.6	0.0	1.6	5.7
3.	UA3	29.5	4.7	0.0	34.2	41.3
4.	UA4	23.9	1.4	0.0	25.2	30.3
5.	UA5	37.9	5.4	0.0	43.2	28.2
6.	UA6	7.2	6.6	0.0	13.8	22.0
7.	UA7	82.8	13.0	0.0	95.8	84.4
8.	UA8	8.9	0.7	0.0	9.7	12.9
9.	UA9	0.0	3.2	0.0	3.2	10.1
10.	UA10	3.0	0.5	0.0	3.5	6.7
11.	UA11	9.9	4.3	0.0	14.2	21.4
12.	UA12	13.0	2.8	0.0	15.9	19.7
13.	UA13	11.9	0.9	0.0	12.8	15.4
14.	UA14	7.8	1.0	0.0	8.8	14.0
15.	UA15	3.9	0.8	0.0	4.7	7.0
16.	UA16	2.8	1.3	0.0	4.1	8.0
17.	UA17	5.6	2.2	0.0	7.8	11.8
18.	UA18	48.8	3.7	0.9	53.4	32.6
19.	UA19	0.4	3.9	0.0	4.2	23.0
20.	UA20	2.5	2.6	0.0	5.1	7.2
21.	UA21	5.1	2.2	0.0	7.4	13.9
	Mean	**15.2**	**2.9**	**0.0**	**18.1**	**20.6**
	SD	**20.2**	**2.9**	**0.2**	**9.7**	**15.4**
	Median	**7.8**	**2.2**	**0.0**	**22.5**	**17.5**
	Min.	**0.0**	**0.0**	**0.0**	**1.6**	**5.7**
	Max.	**82.8**	**13**	**0.9**	**95.8**	**84.4**

Table 3.7 Somali group: Concentration of total arsenic and arsenic species (µg As/g creatinine) in urine samples of Somali group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic
1.	US1	1.2	1.0	0.0	2.1	7.9
2.	US2	0.0	0.9	0.0	0.9	6.1
3.	US3	0.0	0.8	0.0	0.8	3.8
4.	US4	1.0	1.9	0.0	2.9	7.3
5.	US5	4.6	2.9	0.0	7.6	12.5
6.	US6	2.9	1.2	0.0	4.1	12.2
7.	US7	0.8	0.8	0.0	1.7	6.9
8.	US8	0.9	2.0	0.0	2.9	10.2
9.	US9	7.7	1.8	0.0	9.5	14.8
10.	US10	0.0	0.0	0.0	0.0	7.1
11.	US11	0.0	5.0	0.0	5.0	13.3
12.	US12	0.0	0.0	0.0	0.0	5.0
13.	US13	0.0	0.0	0.0	0.0	5.9
14.	US14	0.0	1.7	0.0	1.7	3.9
15.	US15	0.0	3.7	0.9	4.6	5.7
16.	US16	1.5	2.2	0.0	3.8	8.6
17.	US17	1.7	0.9	0.0	2.6	6.1
18.	US18	0.0	0.0	0.0	0.6	2.1
19.	US19	0.0	0.2	0.0	0.2	0.4
20.	US20	0.9	0.7	0.0	1.6	5.2
21.	US21	0.0	0.0	0.0	0.0	3.4
22.	US22	0.0	0.4	0.0	4.7	10.7
	Mean	**1.3**	**1.3**	**0.0**	**2.6**	**7.2**
	SD	**2.0**	**1.3**	**0.2**	**2.5**	**3.8**
	Median	**0.4**	**0.9**	**0.0**	**1.9**	**6.5**
	Min.	**0.0**	**0.0**	**0.0**	**0.0**	**0.4**
	Max.	**7.7**	**5.0**	**0.9**	**9.5**	**14.8**

Table 3.8 White group: Concentration of total arsenic and arsenic species (µg As/g creatinine) in urine samples of White group.

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic
1.	UW1	0.7	2.4	0.0	3.1	19.4
2.	UW2	2.7	6.4	0.0	9.1	25.1
3.	UW3	0.0	0.0	0.0	0.0	11.2
4.	UW4	44.3	0.0	0.0	44.3	33.4
5.	UW5	4.0	2.2	0.0	6.2	12.9
6.	UW6	63.5	10.5	0.0	74.0	65.6
7.	UW7	0.0	5.2	0.0	5.2	21.0
8.	UW8	2.4	1.0	1.6	5.0	9.5
9.	UW9	0.0	6.0	0.0	6.0	16.2
10.	UW10	2.1	4.5	3.1	9.7	17.6
11.	UW11	3.1	6.5	0.0	9.6	17.5
12.	UW12	34.4	2.9	0.0	37.4	49.9
13.	UW13	64.1	0.0	0.0	64.1	71.2
14.	UW14	0.0	5.1	0.0	5.1	8.6
15.	UW15	9.4	11.9	0.0	21.4	34.0
16.	UW16	2.1	2.6	0.0	4.7	7.9
17.	UW17	2.3	5.7	0.0	8.0	10.6
18.	UW18	45.8	9.5	0.0	55.4	42.7
19.	UW19	1.6	2.3	0.0	3.9	6.4
20.	UW20	0.0	2.5	0.0	2.5	8.8
	Mean	**14.1**	**4.4**	**0.2**	**18.7**	**24.5**
	SD	**22.4**	**3.5**	**0.8**	**22.9**	**19.3**
	Median	**2.4**	**3.7**	**0.0**	**7.1**	**17.6**
	Min.	**0.0**	**0.0**	**0.0**	**0.0**	**6.4**
	Max.	**64.1**	**11.9**	**3.1**	**74.0**	**71.2**

Individual data for the three ethnic groups are presented in Fig. 3.6 and 3.7. The data are for total urinary arsenic concentration before (µg /l) and after (µg As/g creatinine) creatinine adjustments are shown in Fig. 3.6 and 3.7, respectively.

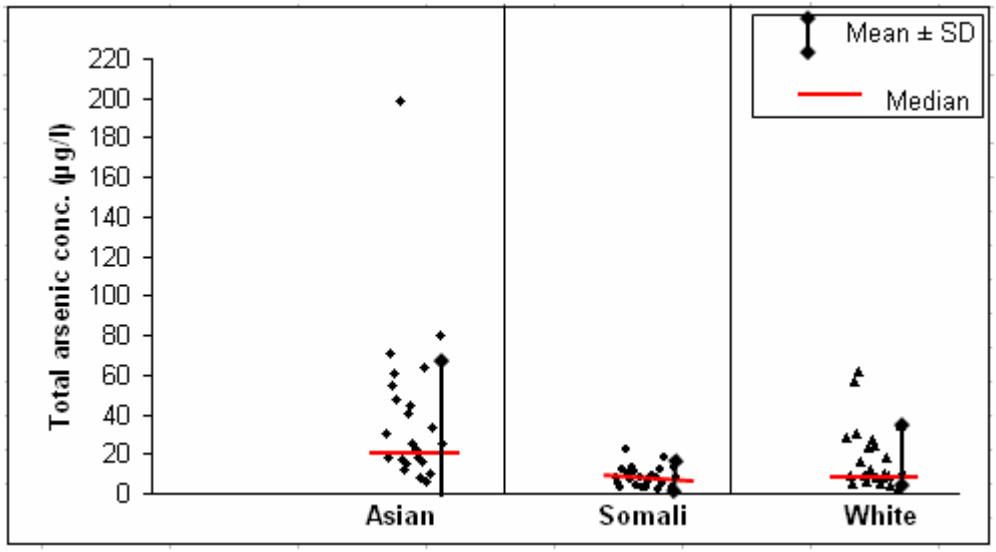

Figure 3.6 Total urinary arsenic concentration (µg/l) before creatinine adjustment for the three ethnic groups (Asian, Somali and White).

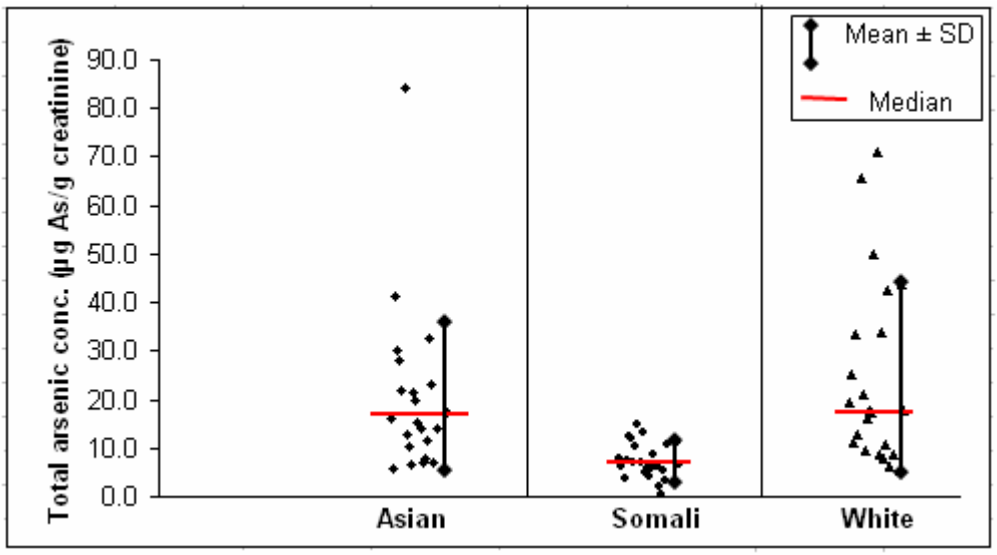

Figure 3.7 Total urinary arsenic concentration (µg As/g creatinine) after creatinine adjustment for the three ethnic groups (Asian, Somali and White).

As can be seen from Figures 3.6 and 3.7, the vast majority of the volunteers within each group had similar arsenic levels in their urine samples. However, few outliers were found in the data of the three ethnic groups before and after creatinine

adjustment in Fig. 3.6 an 3.7, respectively. As shown in Fig. 3.6, two values were found to be outliers in the White group before creatinine adjustment (56.7 and 61.7 µg/l) and one for both the Asian (198.8 µg/l) and the Somali (22.6 µg/l) groups. After creatinine adjustment, the number of outliers were reduced and only one each was found in the Asian (84.4 µg/l) and the White (71.2 µg/l) groups. Few samples showing a high level of total arsenic were observed (Fig. 3.7). The reason for these high values could be due to the volunteers consuming seafood within 3 days prior to sample collection. However, it is important to note that the outliers have no statistically significant effect on the differences between Asian/White compared with the Somali group. Significant differences ($P < 0.05$) between these ethnic groups were detected before and after the exclusion of the outliers (see Table 3.9).

The outliers in the sum of all species among the three groups also followed the same trend as was observed with the total arsenic analysis. The number of outliers were reduced after creatinine adjustment in all groups. A total of three outliers were seen before creatinine adjustment for the Asian group (92.5, 105 and 226 µg/l). In contrast, only one was seen for the Somali group (13.7 µg/l) and two for the White group (69.6 and 75.3 µg/l). After creatinine adjustment, all the outliers disappeared except for the Asian group where two values (53.4 and 95.8 µg As/g creatinine) appear as outliers.

3.3.1.7 Arsenic concentrations in urine samples

In all the three ethnic groups, (Asian, Somali and White) five arsenic species (AB, DMA, As (III), MA and As (V)) were analysed. While As (III) and As (V) were not detected in any single volunteer, AB and DMA were mainly detected in all groups and MA was rarely detected.

Before creatinine adjustment for the Asian group (Table 3.3), total concentration of arsenic varied from 6.5 to 198.8 µg/l, the mean was 38.8 ± 41.5 µg/l. There was one clear outlier with an extremely high value of 198.8 µg/l (Fig. 3.6). The detected arsenic species were AB, DMA and MA, and the mean of sum for all species was 36.4 ± 51.6 µg As/l. Only 3 samples did not show AB and DMA. On the other hand, with exception of one sample, MA was not detected in all the other samples. The range of creatinine concentration was 283.0 – 3136.0 mg/l. After creatinine adjustment (Table 3.6), the mean of total concentration of arsenic in urine was 20.6 ±15.4 µg As/g creatinine, with range of 5.7- 84.4 µg As/g creatinine, one value (84.4 µg As/g creatinine) was considered as an outlier (Fig. 3.7). The mean of sum for all arsenic species was 18.1 ± 9.7µg As/g creatinine with a range of 1.6–95.8 µg As/g creatinine. There were two values (53.4 and 95.8 µg As/g creatinine) that could be considered as outliers.

For the Somali group before creatinine adjustment (Table 3.4), total concentration of arsenic varied from 2.0 to 22.6µg/l, the mean was 8.3 ± 5.2µg/l. Only one sample (22.6 µg/l) appeared to be an outlier (Fig. 3.6). The detected arsenic species were AB, DMA and MA, the mean of sum for all species was 3.2 ± 3.3 µg As/l. The dominant species were AB and DMA. More than 50% of the samples did not show AB, while DMA was detected in all but three samples. MA was detected in only one sample. The range of the creatinine was 577.0 –5060.0 mg/l, the highest value (5060.0 mg/l) was seen in one sample; when the latter was excluded the range was 577.0 – 3804.0 mg/l. After creatinine adjustment (Table 3.7), the mean of total concentration of arsenic in urine was 7.2 ± 3.8 µg As/g creatinine, with a range of 0.4 – 14.8 µg As/g

creatinine, with no outliers. The mean of arsenic species was 2.6 ± 2.5µg As/g creatinine with a range of 0.0– 9.5 µg As/g creatinine.

Urinary arsenic in the White group before creatinine adjustment is presented in Table 3.5. The total concentration of arsenic varied from 4.2 – 61.7 µg/l, with a mean of 18.6 ± 16.2 µg/l. Two samples showed high values (56.7 and 61.7 µg/l) and were considered to be outliers (Fig. 3.6). The detected arsenic species were AB, DMA and MA. The mean of sum for all species was 14.5 ± 21.0 µg As/l. The dominant species were AB and DMA and were detected in all but six samples, while MA was detected in only two samples. The range of creatinine concentration was 159.0 – 2332.0 mg/l. After creatinine adjustment (Table 3.8) the mean of total concentration of arsenic in urine was 24.5 ± 19.3 µg As/g creatinine, with range of 4.2 – 61.7 µg As/g creatinine. One value (71.2 µg As/g creatinine) was considered to be an outlier (Fig. 3.7). The mean of sum for all arsenic species was 18.7 ± 22.9 µg As/g creatinine with a range of 0.0 – 74.0 µg As/g creatinine.

The average total urinary arsenic levels (µg As/g creatinine) in the three ethnic groups are shown in Table 3.6, 3.7 and 3.8 and presented in Fig. 3.8. The average total arsenic was 17.2 ± 16.5 µg As/g creatinine for the three groups combined, while the average among the specific ethnic groups were 24.5 ± 19.3µg As/g creatinine, 20.6 ±15.4 µg As/g creatinine, 7.2 ± 3.8 µg As/g creatinine for the Asian, White and Somali Black-Africans, respectively. Statistical analysis using *t*-test at 95% confidence interval reveals that there is a significant difference ($P < 0.05$) between Asian/White compared with Somali Black-Africans for the total urinary arsenic levels, both before and after inclusion of the outliers, as shown in Table 3.9.

The fact that the sum of the different species detected was lower than the total arsenic is not entirely surprising, and may be explained by the presence of minor species at levels below the detection limit. When speciation analysis is carried out a significant proportion of the species will be below the level of detection of the method (the low total arsenic is sub-divided into various species which will be lower still) and therefore appear to be absent. The lower arsenic levels in these samples lead to greater variability and therefore larger SD values, outliers were also contributed to higher SD, as shown in Table 3.6, 3.7 and 3.8.

The statistical test in this study revealed no significant effect for gender and age on the total of urinary arsenic level.

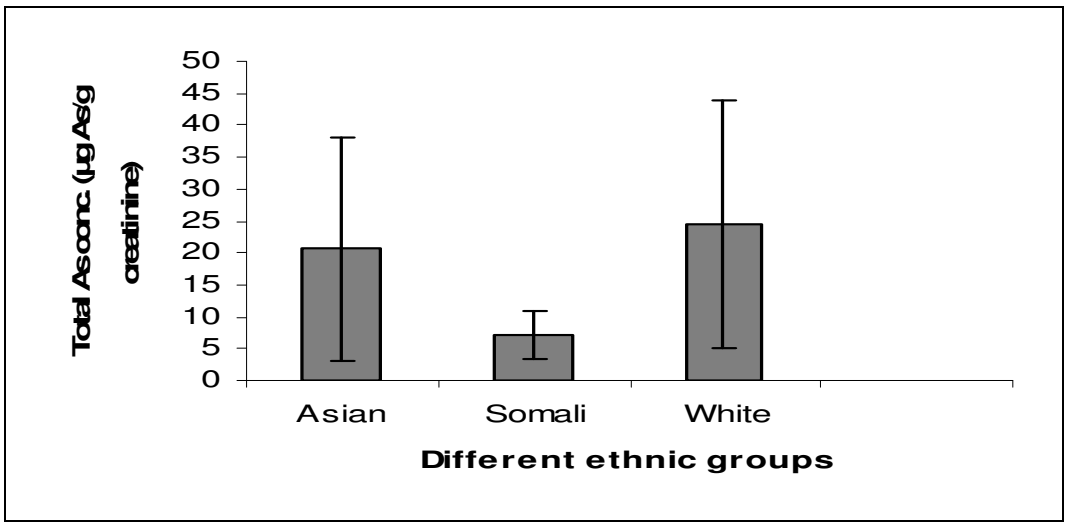

Figure 3.8 Total arsenic concentration (µg As/g creatinine) in urine; among the three ethnic groups: Asian, Somali and Whites.

The results for the arsenic speciation (µg As/g creatinine) in the urine samples from the three ethnic groups determined using HPLC-ICP-MS analysis are shown in Table 3.6, 3.7 and 3.8. Data presented in Table 3.9 shows that the application of *t*-test reveals a significant difference ($P < 0.05$) between Asian and White groups compared to the Somali Black-Africans for AB, DMA, sum of all species and total arsenic. No significant difference was observed for MA ($P > 0.05$). Species percentages are 47 % AB, 50 % DMA and 2 % MA in Somali Black-Africans compared with 83 % AB, 16 % DMA and 1 % MA in Asians and 77 % AB, 22 % DMA and 1 % MA in Whites. Fig. 3.9 summaries how the proportion of the arsenic species (AB, DMA, and MA) vary in the three groups.

Table 3.9 A summary of statistical significance (*P* values) when comparing urinary species (AB, DMA and MMA), sum of all species and total arsenic among the three ethnic groups (Asian, Somali and White), after creatinine adjustment.

Group (n)	AB	DMA	MA	Sum of all species	Total arsenic
Asian (21) vs. White (20)	0.93	0.20	0.34	0.91; 0.27*	0.50; 0.29*
Somali (22) vs. Asian (21)	0.00	0.03	0.68	0.00; 0.00*	0.00; 0.00*
Somali (22) vs. White (20)	0.01	0.00	0.26	0.00; 0.00*	0.00; 0.00*

**P* values after outliers were excluded.

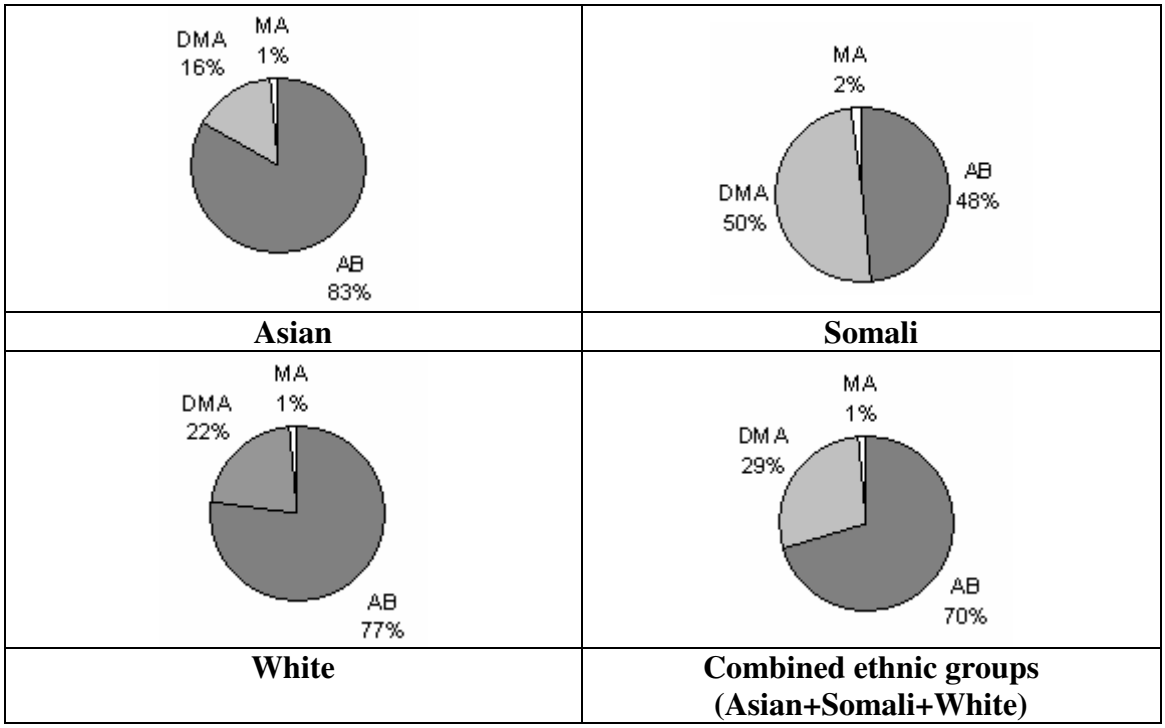

Figure 3.9 Proportion of arsenic species in urine samples of three ethnic groups; Asian, Somali and White.

3. 3. 2 Analysis of hair and fingernail samples

Dates of collection, digestion (using the microwave Prolabo 301, Fig. 2.7) measurements of total arsenic in hair and nail of Asian, Somali and White by using GF-AAS (see Chapter 2, section 2.2.2.7), and location of measurements, are presented in Appendices 3.8,3.9 and 3.10, respectively.

Table 3.10 Asian group: total arsenic concentration (µg/kg) in hair and fingernail samples.

Sample name **Hair**	Total arsenic concentration (µg/kg)	Sample name **Nail**	Total arsenic concentration (µg/kg)
HA1(26)*	52.8	NA1(26)*	139.1
HA2(27)	58.0	NA2(27)	118.9
HA3(28)	103.0	NA3(28)	65.0
HA4(29)	314.5	NA4(29)	181.1
HA5(32)	56.7	NA5(32)	71.3
HA6(33)	94.9	NA6(33)	207.3
HA7(34)	134.8	NA7(34)	151.0
HA8(35)	211.8	NA8(35)	209.3
HA9(36)	26.3	NA9(36)	156.8
HA10(37)	121.4	NA10(37)	239.2
Mean	**117.4**		**153.9**
SD	**87.2**		**57.9**
Median	**99.0**		**153.9**
Min.	**26.3**		**65.0**
Max.	**314.5**		**239.2**

* Numbers between brackets are additional names given to hair and fingernail samples, and apply to the following Tables 3.11 and 3.12. The additional names are read as H26 and N26.

Table 3.11 Somali group: total arsenic concentration (µg/kg) in hair and fingernail samples.

Sample name **Hair**	Total arsenic concentration (µg/kg)	Sample name **Nail**	Total arsenic concentration (µg/kg)
HS1(1)	358.5	NS1(1)	68.5
HS2(2)	154.1	NS2(2)	881.7
HS3(3)	31.7	NS3(3)	121.3
HS4(4)	74.7	NS4(4)	501.4
HS5(5)	197.4	NS5(5)	70.3
HS6(6)	47.9	NS6(6)	897.1
HS7(7)	238.1	NS7(7)	761.8
HS8(8)	98.6	NS8(8)	2806.2
HS9(9)	46.4	NS9(9)	889.4
HS10(10)	128.8	NS10(10)	1971.2
HS11(11)	28.6	NS11(11)	270.0
HS12(12)	106.7	NS12(12)	109.3
HS13(13)	87.0	NS13(13)	1288.7
HS14(14)	108.9	NS14(14)	147.9
HS15(15)	32.4	NS15(15)	62.0
Mean	**116.0**		**723.1**
SD	**91.1**		**798.1**
Median	**98.6**		**501.4**
Min.	**28.6**		**62.0**
Max.	**358.5**		**2806.2**

Table 3.12 **White group:** total arsenic concentration (µg/kg) in hair and fingernail samples.

Sample name **Hair**	Total arsenic concentration (µg/kg)	Sample name **Nail**	Total arsenic concentration (µg/kg)
HW1(30)	29.5	NW1(30)	61.8
HW2(31)	144.1	NW2(31)	265.0
HW3(38)	148.6	NW3(38)	597.2
HW4(39)	244.3	NW4(39)	135.4
HW5(40)	75.3	NW5(40)	208.1
HW6(41)	345.1	NW6(41)	74.1
HW7(42)	78.4	NW7(42)	155.9
HW8(43)	300.0	NW8(43)	221.7
HW9(44)	41.6	NW9(44)	98.5
HW10(45)	66.1	NW10(45)	53.7
HW11 (46)	80.0	NW11(46)	75.2
Mean	**141.2**		**177.0**
SD	**108.4**		**156.5**
Median	**80.0**		**135.4**
Min.	**29.5**		**53.7**
Max.	**345.1**		**597.2**

As shown from Fig 3.10 almost all hair and nail samples among the three ethnic groups are similar in each group, and clustered around their medians. Only few outliers were identified in hair samples and these are as follows: Asian (314 µg/kg), Somali (358 µg/kg) and in nail samples: Somali (2806 and 1971.2 µg/kg) and White (597 µg/kg). These data are also presented Table 3.10, 3.11 and 3.12, for the Asian, Somali and the White groups, respectively. The reason for the very high values (2806 and 1971.2 µg/kg) of the outliers for the Somail group are not clear. Since all the samples had to be used for the analysis it was not possible to carry out additional measurements to check the reproducibility of the data. However, excluding or including the outliers does not result in any significant changes in the statistical outcome (Table 3.13).

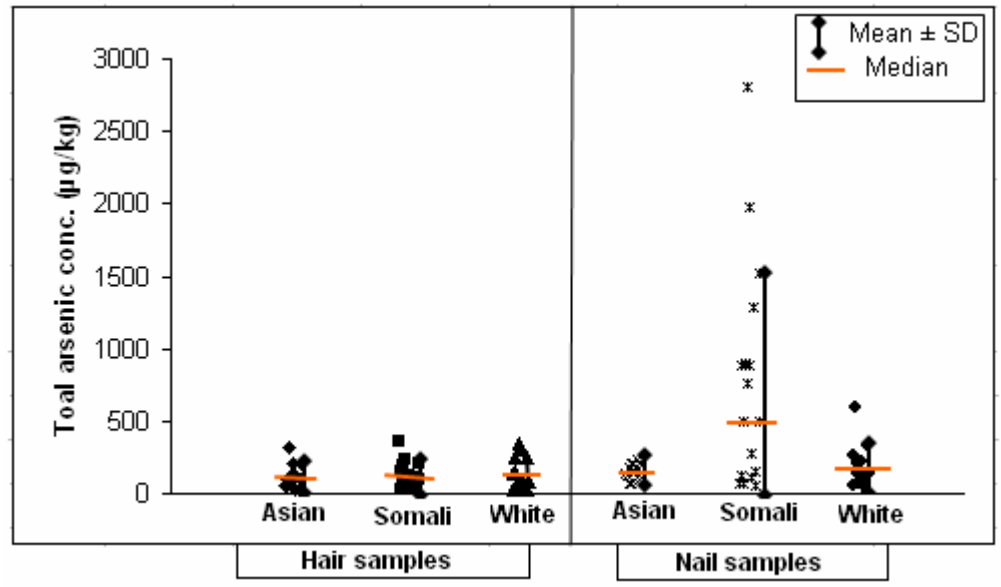

Figure 3.10 Total arsenic concentration (µg/kg) in hair and fingernail samples among the three ethnic groups: Asian, Somali and White.

This study showed the range of total arsenic in hair and fingernails samples was 26.3–358.5 µg/kg and 53.7–2806.2 µg/kg, respectively for the three groups combined.

Fig. 3.11 displays the total arsenic levels of hair and fingernail samples in the three ethnic groups. The average total arsenic in hair and fingernails samples were 124.9 ± 14.2 μg/kg and 351.3 ± 322.2 μg/kg, respectively, for the three groups combined. The averages for each of the ethnic groups were 153.9 ± 57.9 μg/kg, 177.0 ± 156.5 μg/kg and 723.1 ± 798.1 μg/ kg for the Asians, White and Somali Black-Africans, respectively for fingernail samples. Statistical analysis using t-test reveals that there was a significant difference ($P < 0.05$) between Asian/White compared with Somali Black-Africans for the total arsenic levels in fingernails.

For hair samples the average for each of the ethnic groups were 117.4 ± 87.2 μg/kg, 141.0 ± 108.4 μg/kg and 116.0 ± 91.1 μg/kg for the Asians, White and Somali Black-Africans, respectively. However, unlike the fingernail samples there were no significant differences ($P > 0.05$) among the three groups regarding arsenic levels in the hair samples as shown in Table 3.13.

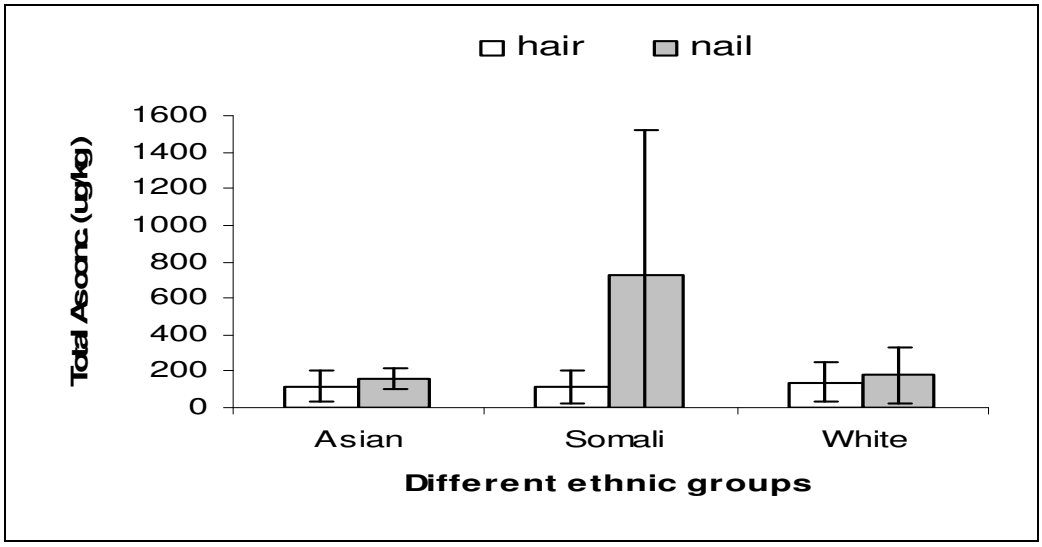

Figure 3.11 The mean total arsenic concentration (µg/kg) in hair and fingernail samples of the three ethnic groups.

Table 3.13 Statistical summary of *P*-values for arsenic concentration (µg/kg) in hair (H) and fingernails (N) samples from Asian, Somali Black Africans and White volunteers. Values in parentheses are number of individuals.

Group (*n*)	*P* -value
Hair	
Somali (H (5) vs Asian (H (10))	0.97; 0.91[a]
Somali (H (5))vs White (H (11))	0.53
Asian (H(10))vsWhite (H (11))	0.59
Fingernails	
Somali (N(15) vs Asian (N (10)	0.04; 0.04[a]
Somali (N (15) vs White (N (11)	0.04
Asian (N(10)vs White (N (11)	0.67

[a] *P*- values associated with excluded outliers

3.4 Discussion

The sample collection, storage and measurement procedures employed in this thesis are similar to what others have used in the scientific literature. Furthermore, a survey of the literature and stability experiments carried out revealed that total arsenic and arsenic species in urine are stable throughout the course of this study. Three days were found to be necessary for the clearance of arsenic originating from seafood from human urine, as indicated from the ingestion of seafood experiments.

The vast majority of the volunteers within each ethnic group have very similar arsenic concentrations in their urine samples (Fig. 3.6 and 3.7). Only few volunteers showed high levels of arsenic in their urine samples making them very different from the vast majority of the individuals. The precise reason for this is not clear. It could be due to these volunteers not strictly adhering to the instructions given with the questionnaire, especially regarding the importance of refraining from the consumption of seafood. However, the overall trend of the study was unaffected, since the exclusion or inclusion of the data from volunteers, appearing as outliers, does not result in any significant changes in the statistical outcome.

As can be seen from Fig 3.6, before creatinine adjustment, and Fig. 3.7 after creatinine adjustment, the outliers either disappeared or were reduced. The reduction of the outliers after creatinine adjustment indicates the importance of creatinine adjustment. This is considered as a means of eliminating different factors that are not associated with arsenic exposure, resulting in a decrease in the number of apparent outliers. It is noteworthy that creatinine adjustment will eliminate different factors that are not related to arsenic exposure such as urine volume and concentration (Hinwood et

al., 2002). Suwazono et al., 2005 and Vahter et al., 2006, also confirmed the importance of creatinine adjustment. When analysing urine samples, the variation in the dilution of urine samples, due to variation of hydration status of the volunteer under study, which are related to fluid intake, temperature etc. These variations can be controlled by creatinine or specific gravity adjustment (Suwazono et al., 2005). This also agrees with what was reported by Vahter et al. (2006) that the concentrations of biomarkers in urine are highly dependent on the dilution of the sample.

There are few studies in the literature that explore the role of ethnicity in arsenic metabolism. Those that do are limited by being restricted to a very small number of individuals (Lai et al., 2004, Hulle et al. 2004), or are based on epidemiological data on populations from different countries (Loffredo et al., 2003) and/or involve the study of exposed populations (Hopenhayn-Rich et al., 1996). Despite these drawbacks, the understanding of arsenic metabolism pathways is progressing thanks to studies investigating speciation of arsenicals in biological samples (Mandal et al., 2004; Steinmaus et al. 2005; Suzuki, 2005).

Comparison of arsenic in urine, hair and fingernail samples of the three ethnic groups

The results of this study reveal that there are significant differences between Somali Black-Africans and Asian/White groups in terms of total arsenic in urine (Table. 3.9) and fingernails (Table 3.13) and also urinary arsenic speciation. The results for the urinary arsenic analysis clearly reveal that the Somali Black-African group has significantly lower levels of total arsenic compared to the White and Asian groups (Fig. 3.8). This difference was not restricted to total arsenic levels because

speciation analysis revealed that differences also exist between these three groups in terms of the levels of different species in urine (Table 3.9). In particular, the proportion of AB is lower and DMA higher in the Somali Black-African group (Fig. 3.9). In order to identify the underlying reason for this difference, hair and fingernail samples from these three groups were also measured (Table 3.10, 3.11 and 3.12). Some This revealed a statistically significant difference between the Somali Black-Africans compared to White and Asians for the fingernail samples with the Somali group having a significant higher total level of arsenic in their fingernails compared to the White and Asian groups (Fig. 3.11). However, the total level of arsenic in the hair samples of the three groups was not significantly different (Table 3.13). There are three possible reasons that may underlie the differences between the groups: (A) dietary; (B) genetic; and (C) a combination of diet and genetics. The possible roles of these factors are discussed below in relation to what has been reported in the literature. However, it is stressed that since this study did not involve a total diet study or genetic analysis, it is not possible to ascertain which of these two factors is responsible for the differences observed in this study.

(A) Role of Diet

The effect of dietary differences cannot be easily answered because this study was not a total diet study and it was not possible to monitor and control the food intake of the volunteers. However, as part of the sample collection process and the associated questionnaire the volunteers were asked to refrain from consuming fish and seafood for at least three days prior to collection of urine. Furthermore, the questionnaire used for hair and fingernail sample collection showed that the most common main meal for the three groups consisted mainly of rice and meat. It was found from this that 89 % of

Asians and 85 % of Somali Black-Africans in the study had a diet that consisted of mainly rice and meat. Significantly, the Somali Black-African diet contained very little vegetable or fish as part of their main meal. Further discussions with the Somali community revealed that their diet is heavily meat based. In contrast, the Asians and White groups appear to have a more balanced diet. In this respect, the Somali diet is very different from the other groups and may partly explain the differences observed.

The importance of diet in arsenic metabolism has been reported by Steinmaus et al. (2005) and showed that individuals with higher protein intake excreted higher levels of DMA in their urine compared with those who had a lower protein intake. It is possible that a protein rich diet provides sufficient quantities of methionine, choline, or cysteine, which are necessary for inorganic arsenic methylation to DMA. It has also been reported that rabbits with low protein dietary intake show a significant decrease in the urinary excretion of DMA (Vahter et al., 1987). Furthermore, Hopenhayn-Rich et al. (1996) concluded that diet may partially play a role in methylation differences, from a study of methylation among populations exposed to arsenic in their drinking water. Thus, literature evidence suggests greater levels of DMA are excreted in the urine of humans and animals that have a higher intake of protein in their diets.

(i) Urine Analysis

In light of the study by Steinmaus et al. (2005), the higher percentage of DMA observed in the urine of Somali Black-African group compared to the other groups suggests that a protein rich diet may explain their arsenic excretion profile compared to the White and Asian groups. However, it is important to point out that this study investigated the relationship between dietary protein intake and arsenic methylation for subjects exposed to inorganic arsenic in their drinking water. In this study, the three

groups reside in the same city and are therefore unlikely to have different levels of inorganic arsenic from their drinking water. Hence, the explanation for the high percentage of DMA in urine of Somali group is either ascribed to the methylation of trace inorganic arsenic that may be contained in their diet or resulting from breakdown of certain organoarsenic compounds in their body. For example, urinary DMA was identified as a major metabolite in human urine after seaweed ingestion (Le et al., 1996; Hulle et al. 2004) as a result of the metabolism of arsenosugars. This was confirmed by Francesconi et al. (2002) where a volunteer ingested a pure arsenosugar and DMA was found to constitute 67% of the total excreted arsenic. However, in the case of this study all the volunteers did not report any consumption of seaweed and all volunteers were asked to refrain from consumption of fish and seafood for 3 days prior to sample collection. Furthermore, the Somali Black-Africans are not known to be consumers of seaweed or shellfish, where arsenosugars are found to occur naturally.

The Somali Black-Africans show the lowest level of AB (48%) in their urine compared to Asians (83%) and White groups (77%). The high levels of AB observed in all three groups is all the more surprising since this compound is known to originate from fish and seafood which the volunteers refrained from eating for at least three days prior to the collection of urine. Other studies have also previously reported that high amounts of AB are excreted in urine (Ritsema et al., 1998; Lai et al. 2004). Studies with 61 healthy volunteers in the Netherlands showed 69.5 % of the total arsenic as AB despite abstaining from seafood for 48 hours (Ritsema et al., 1998). Slow excretion of AB or the ability to metabolise arsenic compounds via a different pathway was offered as a possible explanation for the observation (Lai et al., 2004). This study confirms this finding with a seven fold higher number of volunteers compared to Lai et al. (2004)

although it is still not possible to provide an unequivocal explanation for the presence of AB in urine samples of people refraining from fish and seafood. One explanation may be linked to other sources of AB in the diet, for example it has recently been reported that AB present in chicken (Polatajko et al., 2004) which is widely consumed by the three ethnic groups according to the data from the questionnaire. This may partly explain the reason for the presence of urinary AB because it was detected even amongst volunteers who did not consume fish. However, AB from chicken meat cannot be the sole source because it was also detected in some vegetarian volunteers in this study. Consumption of hidden fish products or other AB containing products cannot be ruled out as reported by Ritsema et al., 1998. Another explanation for the presence of AB could be based on a recent study, which suggests that this compound might be synthesized by humans from trimethylarsine (TMA) (Goessler et al. 1997), although this has been questioned by others (Edmonds 1998). If AB can be synthesized in humans, then this finding would suggest that the lower levels of AB in Somali Black-Africans may be due to them having a lower ability to synthesize this compound. Therefore, the possibility that the human body is capable of metabolizing arsenic compounds to AB must be further investigated.

(ii) Fingernail analysis

Keratin-rich tissues such as hair and nail can accumulate arsenic and this can be used as an indicator of arsenic exposure. Both hair and fingernails are rich in α-keratin which is a fibrous protein containing a high proportion of cysteine residues. The main difference is that the keratins in fingernail contain a higher proportion of this amino acid (up to 22%) compared to hair (10–14%) (Mandal et al., 2003). This study and those of others have shown the level of arsenic in fingernail samples are about 3 times

higher compared to levels in hair. This difference could be attributed to the higher content of cysteine residues in fingernails compared to hair or the slower rate at which nails (0.1 mm/day) grow compared to hair (0.35 mm/day) (Tobin et al., 2005).

The most interesting aspect of the hair and fingernails analysis is the very high level of arsenic observed in fingernails of the Somali Black-African group. They had 4-5 times higher level of arsenic in their fingernail compared to the Asian and White groups. Clearly, Somali fingernails accumulate far higher levels of arsenic compared to the other ethnic groups and this is possibly due to the chemical composition of their fingernails. It is possible their fingernails contain a higher quantity of cysteine rich keratin or some other compound with a high arsenic binding capacity compared to the other groups. From the knowledge of the Leicester-based Somali community and information obtained using the questionnaire it is clear that their diet is more meat based and therefore protein rich, compared to the other groups. Previously it has been reported that there is a positive correlation between arsenic levels in hair and consumption of meat (Saad and Hassanien, 2001). A diet dominated by meat may result in a higher level of cysteine rich keratin in the fingernails of the Somali Black-Africans, thereby favouring greater accumulation of arsenic. However, one cannot rule out the possibility that they possess a higher level of arsenic species, either from metabolism or from their diet, that preferentially deposit in fingernails. As already discussed the Somalis display a higher proportion of total urinary arsenic as DMA compared to the two other ethnic groups. Therefore, it is possible that arsenic in the form of DMA may also be deposited in the fingernails of the Somali ethnic group. This is plausible since it has been previously reported that DMA(III) has greater affinity for fingernail compared to hair (Mandal et al., 2003).

(B) Role of Genetics

The other explanation for the difference in urinary arsenic levels, especially in arsenic speciation, between the two groups could be based on genetic differences associated with arsenic metabolism. Currently three different genes have been suggested to be involved in arsenic biotransformation (Kile et al. 2005 and references therein) , these are As(III) methyltransferase (*CYT19*), purine nucleoside phosphorylase (*PNP*), glutathione-*S*-transferase omega (*GSTO*). Several studies have suggested that there may be a link between genetic polymorphism and arsenic metabolism and these are discussed below.

(i) Urine Analysis

The difference in urinary arsenic levels, especially in arsenic speciation, between the two ethnic groups could be based on genetic differences. A significant difference ($P < 0.05$) was found in the level of one of the methylated species, namely DMA, between the Somali Black-Africans and the White/Asian groups, so it is plausible that a genetic difference underlies this variation in a methylated species. Although there are no data on unexposed population to high arsenic in their drinking water, linking variation in arsenic metabolism with genetic differences, there are several studies with exposed populations which points towards a genetic basis for the difference. For example, it has been reported that there is strong genetic association between polymorphisms of CYT19 and MA: DMA ratio in Mexican children from an arsenic exposed region (Meza et al., 2005). Chung et al. (2002) also reported that methylation differences had a genetic basis, as they found a stronger correlation between arsenic methylation-related phenotypes among siblings than among genetically unrelated individuals. The significantly higher percentage of DMA detected in the

Somali Black Africans in this study requires further investigation to determine if this is due to a genetic basis.

(ii) Fingernail Analysis

As with urinary DMA, the significantly higher level of arsenic observed in the fingernail samples of Somali Black-Africans could also be attributed to genetic factors since a recent study (Kile et al., 2005) reported that the arsenic content in toenails of subjects exposed to inorganic arsenic in their drinking water is related to the GSTT1 gene. Individuals with GSTTl-null genotypes displayed higher levels of total arsenic than those possessing the GSTT1 wild-type gene.

(C) Combined dietary and genetic effects

Another possible explanation for the unusual pattern of arsenic metabolism in Somali Black-Africans could be a combination of diet and genetics. It is possible that a specific dietary habit established over a long period of time, may enhance the activity of particular enzyme(s), which are then inherited by the following generations resulting in metabolic variations between different populations. The Somali Black-Africans are known to have a very protein rich diet, heavily biased towards consumption of animal meat. Following such a diet over many generations may result in over expression of certain genes, linked to metabolism that may explain the unusual arsenic methylation profile observed for this community compared to Asian and White groups. In this context, it is noteworthy that the Somali-Black Africans are recent immigrants to the United Kingdom and therefore less adjusted to a western diet compared to the Whites and Asians. A possible link between dietary habits and arsenic excretion profile, before and after seaweed ingestion, is also apparent from a study reported by Hulle et al. (2004) involving four Chinese and one European. Although the number of individuals

studied by these workers are too few to draw a reliable conclusion, the significantly different excretion profile for the Chinese volunteers compared to the European volunteer may be due to the fact that marine algae has been a major constituent in the diet of Chinese, Japanese and Korean people, which is not the case for the Europeans. However, because this study was not a total diet study and the detailed dietary habits of the three groups were not controlled, it is not possible to unequivocally establish if the difference observed between the groups is due to dietary or genetic factors or if it is a combination of both.

3. 5 Conclusion

All the data from the different studies described above were analysed for each individual within different groups. Only a few individuals showed major deviation from the rest of the population within each ethnic group. However, inclusion or exclusion of these values does not alter the statistical significance of the outcome. The results presented here provides evidence that there is a significant difference in arsenic levels in fingernails and in urine samples of the Somali ethnic group compared to the Asian and White ethnic groups residing in the same city, but not exposed to high concentrations of arsenic. Which of the three factors (dietary, genetic, or a combination of both.) discussed above is responsible for these differences cannot be unequivocally ascertained. Further studies focusing on the role of ethnicity associated with arsenic metabolism among a larger number of healthy individuals with controlled diet from different ethnic communities are needed to resolve this issue.

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Chapter 4
Fasting and Urinary Arsenic Excretion

4.1 Introduction

Millions of people are at risk of various diseases such as cancer and heart disease resulting from chronic arsenic exposure. A historic poisoning has been reported among tens of millions of the population in West Bengal, India and Bangladesh due to high concentration of arsenic in their drinking water (Smith et al., 2000; Chakraborti et al., 2001; Rahman et al., 2001; Mandal and Suzuki, 2002; Charlet and Polya, 2006). The arsenic concentration in groundwater in these regions has been reported to be in the range of 10 –3200 µg/l (Tokunaga et al., 2002; Lindberg et al., 2006; Smedley and Kinniburgh, 2002), exceeding the recommended guideline (10 µg/l) set by the World Health Organization (WHO, 2004). It has been previously reported that arsenic metabolism and toxicity may vary depending on nutritional status of an individual (Mitra et al., 2004). This was investigated among the largest population in the world exposed to arsenic in their drinking water, West Bengal, India and Bangladesh (Mitra et al., 2004). They concluded that low intake of animal protein, folate, calcium and fibre can increase the possibility of arsenic-caused skin lesions, although they could not provide clear evidence of a protective effect of overall protein consumption and vegetables against the development of skin lesions (Mitra et al., 2004; McCarty et al., 2006). Many people in the arsenic-affected regions of India and Bangladesh practice

Ramadan fasting during the daylight hours for one month every year. How this may modulate their pattern of arsenic metabolism has not been previously investigated, but is an issue that requires further investigation.

The general definition of Ramadan fasting is refraining from food and beverages intake for approximately 12 hours a day during the day light hours for a month, which start from dawn to sunset every day. Some changes that are associated with Ramadan fasting includes slowing down of the rate of metabolism, a small reduction in body mass, dehydration and an increase in uric acid in serum (Roky et al., 2004; Toda and Morimoto, 2004). Toda and Morimoto (2004) reported that Ramadan fasting has both positive and adverse effects on healthy individuals, although they suggest that adverse effects are unlikely to continue, or lead to further complications, after Ramadan fasting. They concluded their study by stating that healthy individuals can perform Ramadan fasting without any concern about their health. The subject of fasting and its relationship to health is a rapidly growing field of research. Studies by Mattson (2005) and co-workers have investigated the role of energy intake and meal frequency on health, reporting some positive aspects of intermittent fasting, and caloric restriction, including the suppression of the development of various diseases, and an increase in life span in laboratory animals.

Some biological indicators, such as folic acid and homocysteine, are associated with arsenic metabolism (Chung et al., 2002; Gamble et al., 2005) and these biological parameters undergo significant changes during the fasting. For example, it has been reported that the folate level increases during fasting (Cahill et al., 1998), whereas homocysteine level decreases during Ramadan fasting (Aksungar et al., 2005). Gamble

et al. (2005) reported that arsenic methylation was dependent on folic acid, which is important since inorganic arsenic is methylated to MA and DMA sequentially. Moreover, it was reported that an inadequate folate intake in mice resulted in a decreased biotransformation of urinary arsenic (Spiegel stein et al., 2003). Aksungar et al. (2005) reported that intermittent fasting has beneficial effects on cardiovascular disease, because serum high-density lipoprotein (HDL) levels increased and homocysteine levels decreased during Ramadan fasting. This latter effect could influence arsenic metabolism, because De Kimpe et al. (1999) reported that homocysteine inhibits arsenic methylation. Thus, if Ramadan fasting results in a decrease in the level of homocysteine and an increase in folate, one would expect to see an enhancement of arsenic methylation. The proportion of methylated arsenic species is important since it has been reported that a relatively lower proportion of DMA in human urine is associated with an increased risk of cancers such as bladder and skin cancer among individuals chronically exposed to arsenic (Gamble et al., 2005, and references cited therein). In addition, a high percentage of MA in urine and elevated plasma homocysteine may increase the risk for developing atherosclerosis (Wu et al., 2006).

Toxicity of arsenic in humans is dependent on its chemical form, with As (III) and As (V) being the most toxic forms, MA and DMA are less toxic, and AB which is mainly found in seafood is considered to be non-toxic (Shraim et al., 2001; Vahter et al., 2000; Ritsema et al., 1998). The half-life of inorganic arsenic in the body is two days. Therefore, measuring arsenic in urine can reflect an individual's recent arsenic exposure (Watanabe et al., 2001). Two alternating steps are involved in the pathway for

inorganic arsenic metabolism: reduction of arsenate to arsenite and oxidative methylation of arsenite. These consecutive reduction and oxidative methylation reactions result in DMA as a major arsenic urinary metabolite (Suzuki, 2005). After arsenic exposure, As (V) is reduced to As (III), which is then methylated to MA and DMA (Adair et al., 2005). These methylated metabolites are less toxic than inorganic arsenic, and because of this, the methylation process is considered as a detoxification mechanism (Hsueh et al., 1998). However, MA (III) and DMA (III) produced through the methylation process are considered more toxic than inorganic arsenic (Hsueh et al., 1998). Generally, MA and DMA are the only metabolites arising from the methylation process and appear in urine, although a recent publication reported MA (III) and DMA (III) in urine of chronically exposed populations to inorganic arsenic (Valenzuela et al., 2005). Fasting is one of the factors that may have an influence on arsenic species distribution, since it has been associated with changes in some important biological parameters that have an effect on arsenic metabolism.

The aim of this study was to investigate whether Ramadan fasting - abstaining from food and fluid for a period of about 12 hours - results in a modification of arsenic metabolism, as investigated through speciation analysis of urine samples. This study is potentially important since many people in arsenic-affected regions of India and Bangladesh practice Ramadan fasting during the day light hours for one month every year. The study involved recruitment of 29 Ramadan fasting volunteers living in Leicester, UK. Urine samples were monitored for their arsenic levels at the beginning and the end of an approximately 12h long fast.

4.2 Experimental

This section includes the following sub-sections: chemical reagents, instrumentation, samples collection, quality control, creatinine determination and statistical analysis.

4.2.1 Chemicals and reagents

The chemicals reagents used for experiments related to this Chapter was same to that reported in Chapter 3 (section 3.2.2.)

4.2.2 Instrumentation

The instrumentation and analysis used for experiments related to this Chapter is described in Chapter 2 (sections 2.2.4 and 2.2.5)

4.2.3 Samples collection and preparation

Urine sample collection and storage were carried out similar as reported in Chapter 3 (section 3.2.1.2). Samples were collected from 29 Ramadan fasting (RF) volunteers. Each volunteer gave two samples. One sample was collected at the beginning of the fasting period (RF1), first morning void urine, and the second sample was collected at the end of the fasting period (RF2), first sunset void urine. Dates of urine samples collection were spread in the middle (24/10/2004 – 09/11/204) of the Ramadan, for a single volunteer and the RF group, one week after the beginning (15/10/2004) of the month and one week before the end (14/11/2004) of the fasting month. The collected samples from a single fasting volunteer were over five

consecutive days. The RF group were from Leicester, UK [mean age 31.9 years (one individual did not report his age); 3 women and 26 men]. The ethnicity background of the volunteers was mixed and composed of nine Asian or Asian British, 10 Black or Black British, seven Middle Eastern and three North African. All volunteers were asked to refrain from eating fish and seafood for three days prior to sample collection, and to complete a questionnaire, which gathered information on age, gender and ethnicity along with lifestyle (all this information is detailed in Appendix 4.1). All procedures regarding ethics approval, samples collection, normality test, storage, and sample pre-treatment were same as reported in Chapter 2 (section 2.2.1.2) and Chapter 3 (section 3.2.1).

Appendices 4.2 and 4.3 show the dates of collection and dates of measurements and locations for total arsenic and arsenic speciation for a single fasting volunteer and Ramadan fasting volunteers (n = 29), respectively.

Data for the non-fasting group (NF) was taken from Chapter 3.

4.2.4 Quality control and method validation

The quality control and method validation procedures related to this Chapter is same to what has been reported in Chapter 2 (sections 2.2.4 and 2.2.5).

4.2.5 Determination of creatinine

The creatinine measurement for this Chapter is as described in Chapter 2 (section 2.2.1.3).

4.2.6 Statistical analysis

Differences (95% confidence level) between the two groups (RF1 and RF2) in terms of arsenic species and total arsenic levels in urine samples were evaluated by using Paired Student's *t*-test, since all the participants were sampled twice. The difference between RF1 and RF2 for MA in terms of level and percentage was also tested by using the same test (Paired Student's *t*-test), by assigning half of the lowest detected concentration or calculated percentage of MA to not-detected MA, because MA was not detected in many samples. The Student's *t*-test was used to evaluate the influence (95% confidence level) of age and gender. The Kolmogorov-Smirnov test (KS-test) was used for not normally distributed data. Moreover, for analysis of MA frequency of detection, estimates and 95% confidence intervals for the odds ratio (OR) were calculated. The frequency distribution (*P*-value) for data sets were calculated using the Fisher Exact test.

4.3 Results

All the urine samples investigated were found to be normal according to the results of test strip analysis. The pH of all the 58 urine samples studied was found to be in the range of 5 to 8. This is within the normal range (4.5 – 8.0) expected for human urine (Chen et al., 2002).

Table 4.1 shows arsenic concentration before creatinine adjustment, while Table 4.2 shows the concentrations after creatinine adjustment. The levels of arsenic in RF1 urine

samples of a single fasting volunteer collected over five consecutive days was in the range of 7.6 – 25.0 µg/l with a mean of 13.1 ± 7.1 µg/l before creatinine adjustment (Table 4.1) and the range was 5.8 – 18.0 µg/g creatinine with a mean of 10.0 ± 5.1 µg/g creatinine, after creatinine adjustment (Table 4.2). For RF2 urine samples, the range was 15.9 – 36.8 µg/l with a mean of 20.9 ± 9.0 µg/l before creatinine adjustment (Table 4.1) and the range was 4.6 – 14.5 µg/g creatinine after creatinine adjustment with a mean of 9.2 ± 4.5 µg/g creatinine (Table 4.2).

The percentages of arsenic species for a single volunteer were calculated after creatinine adjustment (Table 4.2). For RF1 the percentages were 68% AB and 29% DMA, compared with 60% AB and 34% DMA for RF2. There was a decrease in the percentage of AB and an increase in the percentage of DMA observed in RF2 urine samples. A trend of increasing percentage of methylated arsenic toxic species (DMA) compared to AB for RF2 samples has detected. However, there was no difference between RF1 and RF2 in terms of total arsenic levels.

Table 4.1 Concentrations of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) in urine samples from a single fasting volunteer.

Sample No.	Sample name **RF1**	AB	DMA	Sum of all species	Total arsenic (µg/l)	Creatinine (mg/l)
1.	UNC1F1	0.6	1.0	1.6	8.6	1404
2.	UNC1F2	0.9	0.8	1.7	7.6	423
3.	UNC1F3	10.1	1.7	11.8	25.0	2106
4.	UNC1F4	3.8	0.8	4.6	13.9	1721
5.	UNC1F5	<LOD	<LOD	<LOD	10.2	1766
	Mean	**3.1**	**0.9**	**4.9**	**13.1**	**1484**
	SD	**4.2**	**0.6**	**4.8**	**7.1**	**643.0**
	Median	**0.9**	**0.8**	**3.2**	**10.2**	**1721**
	Min.	**0**	**0**	**1.6**	**7.6**	**423**
	Max.	**10.1**	**1.7**	**11.8**	**25**	**2106**
	RF2					
1.	UC1F1	<LOD	3.0	3.0	15.9	3487
2.	UC1F2	4.6	2.1	6.7	36.8	2773
3.	UC1F3	1.0	2.3	3.3	18.2	3068
4.	UC1F4	4.1	1.9	6.0	17.1	2196
5.	UC1F5	2.5	2.0	4.5	16.3	1121
	Mean	**2.4**	**2.3**	**4.7**	**20.9**	**2529**
	SD	**2.0**	**0.4**	**1.6**	**9.0**	**917.0**
	Median	**2.5**	**2.1**	**4.5**	**17.1**	**2773**
	Min.	**<LOD**	**1.9**	**3.0**	**15.9**	**1121**
	Max.	**4.6**	**3**	**6.7**	**36.8**	**3487**

Table 4.2 Concentrations of total arsenic and arsenic species (µg As/g creatinine) in urine samples from a single fasting volunteer at two time points (RF1 and RF2).

Sample No.	Sample name **RF1**	AB	DMA	Sum of all species	Total arsenic
1.	UNC1F1	0.4	0.7	1.1	6.1
2.	UNC1F2	2.1	1.9	4.0	18.0
3.	UNC1F3	4.8	0.8	5.6	11.9
4.	UNC1F4	2.2	0.5	2.7	8.1
5.	UNC1F5	0.0	0.0	0.0	5.8
	Mean	**1.9**	**0.8**	**2.7**	**10.0**
	SD	**1.9**	**0.7**	**2.2**	**5.1**
	Median	**2.1**	**0.7**	**2.7**	**8.1**
	Min.	**<LOD**	**<LOD**	**<LOD**	**5.8**
	Max.	**4.8**	**1.9**	**5.6**	**18.0**
	RF2				
1.	UC1F1	0.0	0.9	0.9	4.6
2.	UC1F2	1.7	0.8	2.4	13.3
3.	UC1F3	0.3	0.7	1.1	5.9
4.	UC1F4	1.9	0.9	2.7	7.8
5.	UC1F5	2.2	1.8	4.0	14.5
	Mean	**1.2**	**1.0**	**2.2**	**9.2**
	SD	**1.0**	**0.4**	**1.3**	**4.5**
	Median	**1.7**	**0.9**	**2.4**	**7.8**
	Min.	**<LOD**	**0.7**	**0.9**	**4.6**
	Max.	**2.2**	**1.8**	**4.0**	**14.5**

Fig. 4.1 and 4.2 show HPLC-ICP-MS chromatograms of arsenic speciation in two urine samples for RF1 and RF2 time point, respectively for a single fasting volunteer. A chloride peak was observed in both Figures with an increase in Fig. 4.2, which suggests more chloride is observed in fasting urine samples that increases at the end of the fasting period.

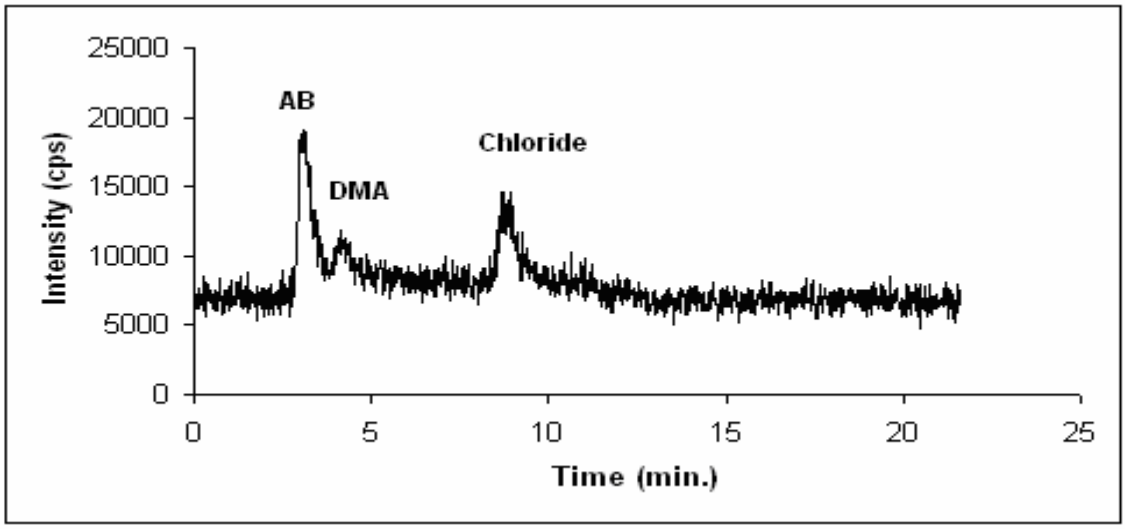

Figure 4.1 HPLC-ICP-MS chromatogram for speciation of arsenic in the urine of a single fasting volunteer RF1 (UC1NF3).

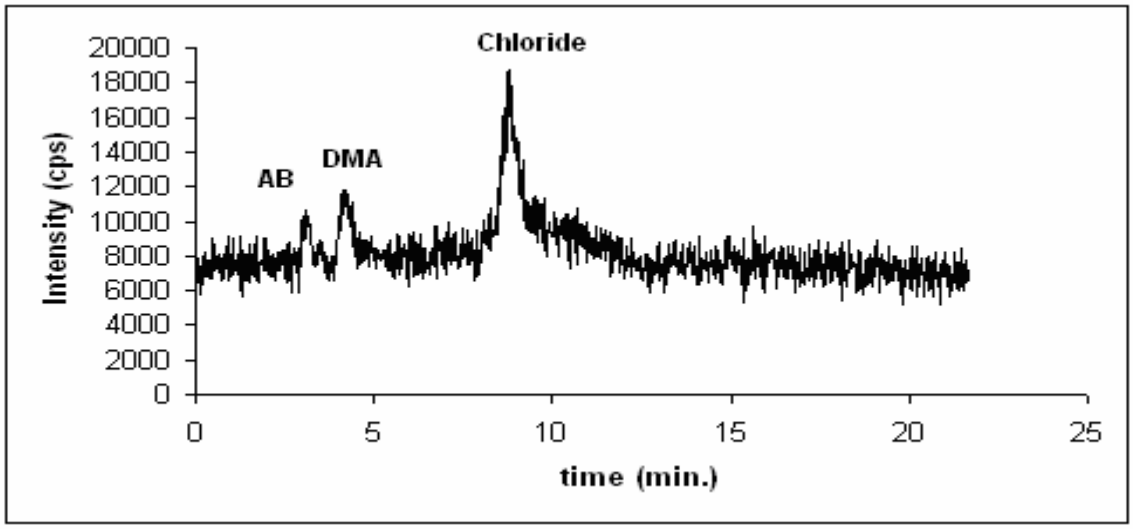

Figure 4.2 HPLC-ICP-MS chromatogram for speciation of arsenic in the urine of a single fasting volunteer RF2 (UC1F3).

The levels of arsenic in urine samples of the time points RF1 and RF2 samples for 29 volunteers of Ramadan fasting group are shown in Table 4.3 and 4.4, respectively, before creatinine adjustment. In addition, the levels of arsenic after creatinine adjustment for the sample of the two time points, RF1 and RF2 for the 29 volunteers, are shown in Table 4.5 and 4.6, respectively. Total arsenic in RF1 was in the range of 5.3 – 83.6 µg/l with a mean of 24.9 ± 19.9 µg/l before creatinine adjustment, and 2.4 – 46.4 µg/g creatinine with a mean of 13.3 ± 12.4 µg/g creatinine after creatinine adjustment. While total arsenic in RF2 was in the range of 7.0 –136.8µg/l with a mean of 30.2 ± 27.6 µg/l before creatinine adjustment, and1.8 –52.3 µg/g creatinine with a mean of 11.5 ± 9.9 µg/g creatinine after creatinine adjustment.

Table 4.3 Concentrations of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) urine samples of the fasting group at the beginning of the fasting period (RF1).

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic	Creatinine (mg/l)
1.	UNF1	5.4	7.5	1.2	14.1	5.3	804
2.	UNF2	4.8	3.2	1.5	9.5	20.2	3090
3.	UNF3	13.5	3.9	1.6	19	33.8	2196
4.	UNF4	4.0	4.2	1.3	9.5	12.2	1472
5.	UNF5	8.0	3.3	1.2	12.5	16.4	2660
6.	UNF6	39.0	19.4	2.2	60.6	83.6	3396
7.	UNF7	8.0	0.8	0	8.8	14.2	1211
8.	UNF8	7.8	6.4	0	14.2	12.2	1234
9.	UNF9	8.8	6.5	2.3	17.6	30.9	3328
10.	UNF10	6.1	1.1	0	7.2	25.2	1189
11.	UNF12	11.9	4.1	3.1	19.1	25.7	985
12.	UNF13	12.2	3.4	0	15.6	16.3	1223
13.	UNF14	2.6	6.3	2.2	11.1	12.7	1041
14.	UNF15	8.4	3.3	0	11.7	16.1	532
15.	UNF16	69.5	15.9	0.8	86.2	78.9	1845
16.	UNF17	47.5	8.8	0	56.3	51.0	1245
17.	UNF19	0	3.3	1.2	4.5	7.8	1381
18.	UNF21	4.7	1.5	0	6.2	9.2	589
19.	UNF22	5.2	2.6	0	7.8	10.1	951
20.	UNF24	7.3	8	0.8	16.1	15.5	894
21.	UNF25	8.1	1.6	0	9.7	15.5	2287
22.	UNF26	8.0	2.9	0	10.9	13.1	238
23.	UNF27	4.2	1.7	0	5.9	10.5	724
24.	UNF28	0.9	1.9	0	2.8	9.1	498
25.	UNF29	12.9	6.6	0	19.5	37.1	2004
26.	UNF30	3.9	3.6	0	7.5	13.0	679
27.	UNF32	13.3	3.7	2	19	46.0	2015
28.	UNF33	5.6	3.0	0	8.6	42.3	2117
29.	UNF34	9.4	<LOD	0	9.4	38.3	1789
	Mean	**11.8**	**4.8**	**0.5**	**13.4**	**24.9**	**1504**
	SD	**14.9**	**4.2**	**0.7**	**12.3**	**19.9**	**860**
	Median	**8**	**3.4**	**0.0**	**9.4**	**16.1**	**1234**
	Min.	**0.0**	**0.0**	**0.0**	**3.1**	**5.3**	**238**
	Max.	**69.5**	**19.4**	**3.1**	**46.7**	**83.6**	**3396**

Table 4.4 Concentrations of total arsenic (µg/l), arsenic species (µg As/l) and creatinine (mg/l) in urine samples of the fasting group, at the end of the fasting period (RF2).

Sample No.	Sample name	AB	DMA	MA	Sum of all species	Total arsenic	Creatinine (mg/l)
1.	UF1	8.4	17.8	2	28.2	64.4	4403
2.	UF2	10.0	2.0	0.8	12.8	20.1	3022
3.	UF3	9.5	5.1	1.2	15.8	27.8	4030
4.	UF4	6.6	5.0	1.8	13.4	25.1	2106
5.	UF5	0.8	1.0	0	1.8	7.6	985
6.	UF6	87.5	17.5	2.2	107.2	136.8	2049
7.	UF7	18.2	2.3	0	20.5	33.8	1709
8.	UF8	23.4	15.1	3.1	41.6	35.3	3509
9.	UF9	8.8	6.5	1.8	17.1	26.0	1936
10.	UF10	0.6	2	0	2.6	9.1	1460
11.	UF12	12.4	3.9	1.1	17.4	27.1	860
12.	UF13	9.4	5.0	1.1	15.5	18.3	1211
13.	UF14	7.4	8.6	1.5	17.5	22.3	951
14.	UF15	20.0	7.1	0.9	28	33.1	1743
15.	UF16	14.4	4.9	0	19.3	21.2	2083
16.	UF17	13.7	6.4	1	21.1	22.1	1721
17.	UF19	1.4	11.6	3.6	16.6	23.2	1245
18.	UF21	1.1	3.4	0	4.5	11.5	1675
19.	UF22	1.4	3.1	0	4.5	7.2	272
20.	UF24	17.5	20.5	4.1	42.1	48.0	1562
21.	UF25	8.6	0.8	0	9.4	12.8	702
22.	UF26	11.0	4.6	1.1	16.7	22.5	1789
23.	UF27	22.0	8.0	0.8	30.8	7.0	3384
24.	UF28	3.1	3.2	0	6.3	16.5	1823
25.	UF29	33.6	15.4	0	49	96.1	2932
26.	UF30	3.9	4.1	0	8	19.3	1664
27.	UF32	7.1	6.0	2.3	15.4	42.2	2151
28.	UF33	3.4	3.9	1.3	8.6	25.3	951
29.	UF34	2.2	<LOD	0	2.2	14.0	623
	Mean	**12.7**	**6.7**	**1.1**	**20.5**	**30.2**	**1881.1**
	SD	**16.4**	**5.5**	**1.2**	**20.6**	**27.6**	**1017**
	Median	**8.8**	**5**	**1**	**16.6**	**22.5**	**1721**
	Min.	**0.6**	**0**	**0**	**1.8**	**7.0**	**272**
	Max.	**87.5**	**20.5**	**4.1**	**107.2**	**136.8**	**4403**

Table 4.5 Concentrations of total arsenic and arsenic species (µg As/g creatinine) in urine samples of the fasting group (RF1).

Sample No.	Sample name (**RF1**)	AB	DMA	MA	Sum of all species	Total arsenic
1.	UNF1	6.7	9.3	1.5	17.5	6.6
2.	UNF2	1.6	1.0	0.5	3.1	6.5
3.	UNF3	6.1	1.8	0.7	8.7	15.4
4.	UNF4	2.7	2.9	0.9	6.5	8.3
5.	UNF5	3.0	1.2	0.5	4.7	6.2
6.	UNF6	11.5	5.7	0.6	17.8	24.6
7.	UNF7	6.6	0.7	0.0	7.3	11.7
8.	UNF8	6.3	5.2	0.0	11.5	9.9
9.	UNF9	2.6	2.0	0.7	5.3	9.3
10.	UNF10	5.1	0.9	0.0	6.1	21.2
11.	UNF12	12.1	4.2	3.1	19.4	26.1
12.	UNF13	10.0	2.8	0.0	12.8	13.3
13.	UNF14	2.5	6.1	2.1	10.7	12.2
14.	UNF15	15.8	6.2	0.0	22.0	30.3
15.	UNF16	37.7	8.6	0.4	46.7	42.8
16.	UNF17	38.2	7.1	0.0	45.2	41.0
17.	UNF19	0.0	2.4	0.9	3.3	5.6
18.	UNF21	8.0	2.5	0.0	10.5	15.6
19.	UNF22	5.5	2.7	0.0	8.2	10.6
20.	UNF24	8.2	8.9	0.9	18.0	17.3
21.	UNF25	3.5	0.7	0.0	4.2	6.8
22.	UNF26	33.6	12.2	0.0	45.8	55.0
23.	UNF27	5.8	2.3	0.0	8.1	14.5
24.	UNF28	1.8	3.8	0.0	5.6	18.3
25.	UNF29	6.4	3.3	0.0	9.7	18.5
26.	UNF30	5.7	5.3	0.0	11.0	19.1
27.	UNF32	6.6	1.8	1.0	9.4	22.8
28.	UNF33	2.6	1.4	0.0	4.1	20.0
29.	UNF34	5.3	0.0	0.0	5.3	21.4
	Mean	**9.0**	**3.9**	**0.5**	**13.4**	**18.3**
	SD	**10.1**	**3.1**	**0.7**	**12.3**	**15.6**
	Median	**6.1**	**2.8**	**0.0**	**9.4**	**11.8**
	Min.	**0.0**	**0.0**	**0.0**	**3.1**	**5.6**
	Max.	**38.2**	**12.2**	**0.5**	**13.4**	**55.0**

Table 4.6 Concentrations of total arsenic and arsenic species (µg As/g creatinine) in urine samples of the fasting group (RF2).

Sample No.	Sample name (**RF2**)	AB	DMA	MA	Sum of all species	Total arsenic
1.	UF1	1.9	4.0	0.5	6.4	14.6
2.	UF2	3.3	0.7	0.3	4.2	6.7
3.	UF3	2.4	1.3	0.3	3.9	6.9
4.	UF4	3.1	2.4	0.9	6.4	11.9
5.	UF5	0.8	1.0	0.0	1.8	7.7
6.	UF6	42.7	8.5	1.1	52.3	66.8
7.	UF7	10.6	1.3	0.0	12.0	19.8
8.	UF8	6.7	4.3	0.9	11.9	10.1
9.	UF9	4.5	3.4	0.9	8.8	13.4
10.	UF10	0.4	1.4	0.0	1.8	6.2
11.	UF12	14.4	4.5	1.3	20.2	31.5
12.	UF13	7.8	4.1	0.9	12.8	15.1
13.	UF14	7.8	9.0	1.6	18.4	23.4
14.	UF15	11.5	4.1	0.5	16.1	19.0
15.	UF16	6.9	2.4	0.0	9.3	10.2
16.	UF17	8.0	3.7	0.6	12.3	12.8
17.	UF19	1.1	9.3	2.9	13.3	18.6
18.	UF21	0.7	2.0	0.0	2.7	6.9
19.	UF22	5.1	11.4	0.0	16.5	26.5
20.	UF24	11.2	13.1	2.6	27.0	30.7
21.	UF25	12.3	1.1	0.0	13.4	18.2
22.	UF26	6.1	2.6	0.6	9.3	12.6
23.	UF27	6.5	2.4	0.2	9.1	2.1
24.	UF28	1.7	1.8	0.0	3.5	9.1
25.	UF29	11.5	5.3	0.0	16.7	32.8
26.	UF30	2.3	2.5	0.0	4.8	11.6
27.	UF32	3.3	2.8	1.1	7.2	19.6
28.	UF33	3.6	4.1	1.4	9.0	26.6
29.	UF34	3.5	0.0	0.0	3.5	22.5
	Mean	**7.0**	**3.9**	**0.6**	**11.5**	**17.7**
	SD	**7.9**	**3.3**	**0.8**	**9.9**	**12.5**
	Median	**5.1**	**2.8**	**0.5**	**9.3**	**14.6**
	Min.	**0.4**	**0.0**	**0**	**1.8**	**2.1**
	Max.	**42.7**	**13.1**	**2.9**	**52.3**	**66.8**

Fig. 4.3 and 4.4 show representative HPLC-ICP-MS chromatograms of arsenic speciation in two urine samples for RF1 and RF2 time point, respectively, for one volunteer from the fasting group. Appendix 4.4 includes more chromatograms for blank, standard solutions, certified urine sample (CRM NIES No.18) and real samples. The results of the CRM NIES No. 18 were as follows: AB 69.5 ± 4.8µg As/l and DMA 33.5 ± 1.3 µg As/l; the certified values were 69 ± 12 µg/l and 36 ± 9µg/l, respectively. The reproducibility of the method was also validated by measuring 10 µg As/l standard mixture of the five arsenic species after each six runs. The reproducibility (n =3) was 10.2 ± 0.2µg As/l (1.5% RSD) for AB, 10.1 ± 0.3 µg As/l (2.6% RSD) for DMA, 10.2 ± 0.3 µg/l (1.7% RSD) for As (III), 10.2 ± 0.1µg As/l (1.2% RSD) for MA and 10.0 ± 0.1 µg/l (1.0% RSD) for As (V).

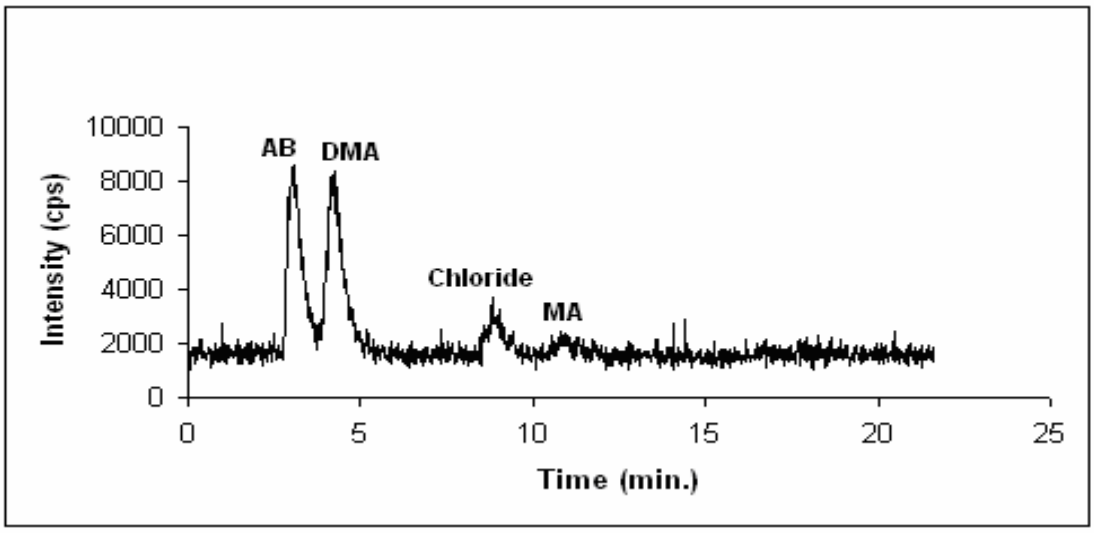

Figure 4.3 HPLC-ICP-MS chromatogram for speciation of arsenic in the urine sample of a fasting volunteer RF1 (UNF24).

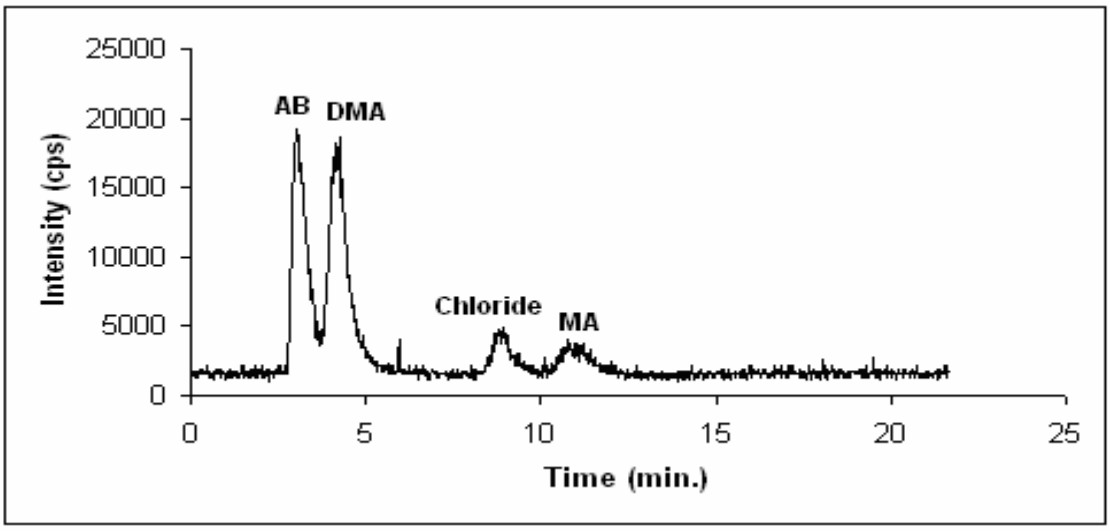

Figure 4.4 HPLC-ICP-MS chromatogram for speciation of arsenic in the urine sample of a fasting volunteer RF2 (UF24).

Total arsenic levels of all the volunteers - before and after creatinine adjustment - for the fasting group are presented in Fig. 4.5. This reveals only few outliers. There were two (83.6, 78.9 µg/l) and three (55.0, 42.8, 41.0 µg As/g creatinine) for RF1 before and after creatinine adjustment, respectively. For RF2 there were three (136.8, 96.1, 64.4 µg/l) and one (66.8 As/g creatinine) outliers before and after creatinine adjustment, respectively. These values for both RF1 and RF2 appeared as outliers due to their very high arsenic level compared to the vast majority of the other volunteers. However, the exclusion or inclusion of these outliers does not influence the statistical significance (P = 0.67 and 0.86 respectively) of the analysis when comparing RF1 with RF2.

The most plausible explanation for the outliers is that these volunteers did not adhere to the strict request to avoid consumption of seafood within 3 days prior to sample collection. Despite these outliers, there were no statistical significant differences ($P > 0.05$) between RF1 and RF2 for total arsenic levels. The combined levels of total arsenic (18.0 ± 12.1 µg/g creatinine) for RF group was also compared with the mean arsenic level (17.2 ± 16.5µg/g creatinine) for the non-fasting group (NF, ethnic group: Asian, Somali and White that was studied in Chapter 3). There is no statistically significant difference ($P > 0.05$) between these two groups, which suggests that Ramadan fasting has no effect on total urinary arsenic levels.

The influence of gender and age on total arsenic in urine samples (RF1 and RF2) was tested . However, this did not reveal any significant influence.

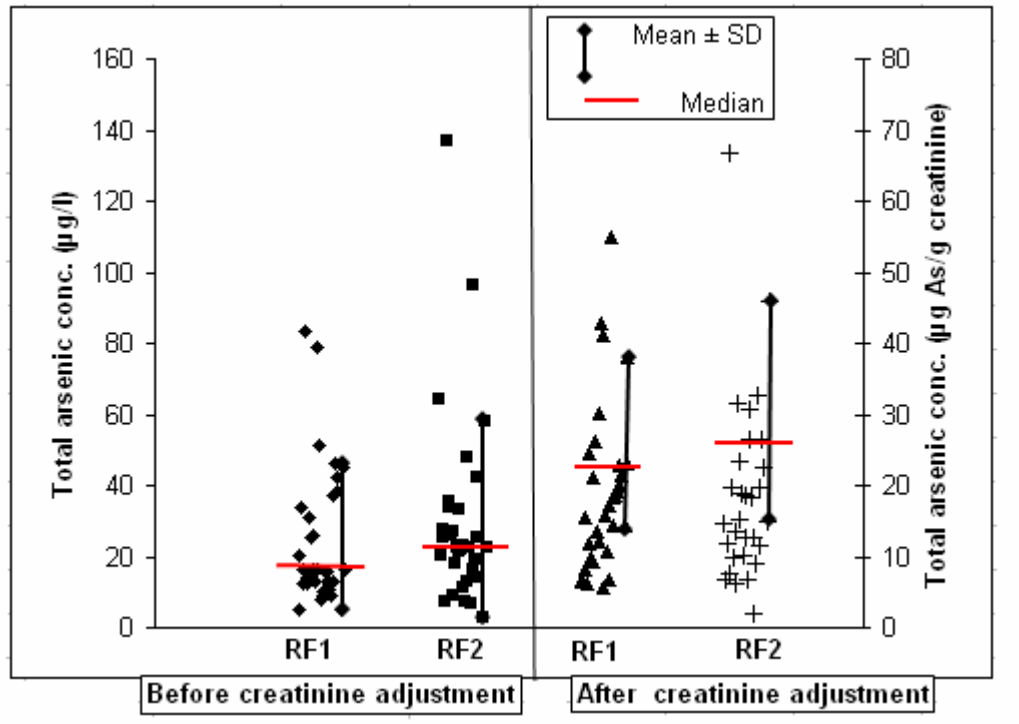

Figure 4. 5 Urinary arsenic concentration (µg /l) before and after (µg As/g creatinine) creatinine adjustment for RF1 and RF2 samples.

The measured species percentages in the two different sample types for the 29 volunteers were 67% AB and 29% DMA and 4% MA in RF1, compared to 61% AB, 34% DMA and 5% MA in RF2 samples. These percentages of arsenic species in fasting group for the two time points RF1 and RF2, combined fasting group (RF) and non-fasting group (NF) are presented in Fig. 4.6. The combined percentages of these species among the fasting group were 64% AB, 31% DMA and 5% MA, compared to the combined percentages of these species among the non-fasting group being 70% AB, 29% DMA and 1% MA. This shows that the detected toxic organoarsenic species -

DMA and MA – are present in higher percentage amongst the fasting group compared to the non-fasting group.

Methylarsonate was detected in 53% (31 out of 58) of the fasting samples compared to only 6% (4 out of 63) of the non-fasting samples. Differences in MA between fasting and non-fasting groups was assessed by comparison of the frequency of detection. The odds ratio (OR) was 12.0 (95%CI 3.8 to 37.4), with $P < 0.001$. This reveals that MA in the fasting group is almost 12 times as likely to be detected as among non-fasting group. In the fasting group (RF1 and RF2) MA was detected in 62% (18 out of 29) of RF2 samples compared to only 45% (13 out of 29) of RF1 samples. Differences in MA between RF2 and RF1 groups was assessed by comparison of frequency of detection. The odds ratio (OR) was 2.0 (95%CI 0.7 to 5.7), with a $P > 0.001$. This reveals that MA at the end of the fast (RF2) is almost twice as likely to be detected as at the beginning of the fast (RF1).

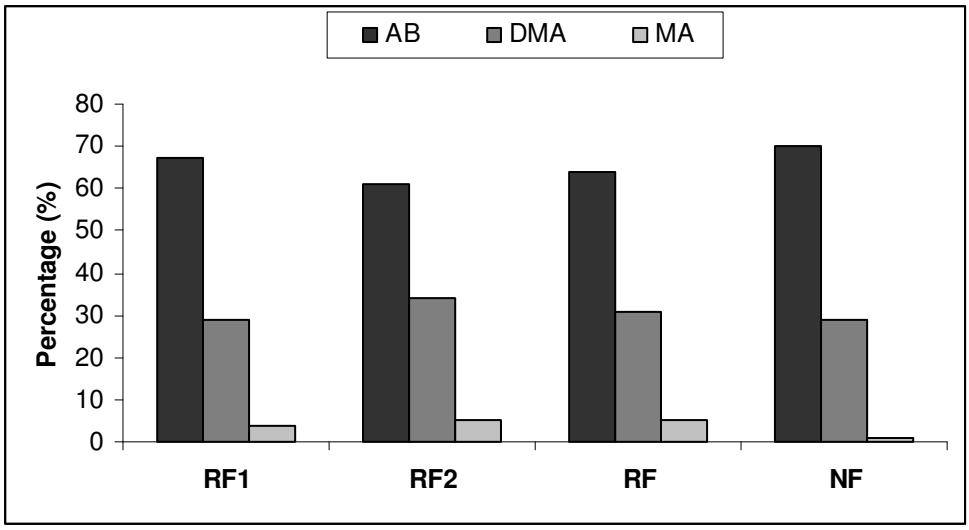

Figure 4.6 Percentages of arsenic species in urine of fasting group for two time points RF1, RF2, combined fasting group (RF) and non-fasting (NF). The data for the NF group is taken from Chapter 3.

Table 4.7 shows *P*-values presented for a single fasting volunteer as well as for the fasting group and the non-fasting group. Significant differences ($P < 0.05$) were observed in both the levels and percentages of MA between the fasting and the non-fasting group, while no significant differences ($P > 0.05$) were found by comparing the total arsenic levels or the arsenic species between the fasting and the non-fasting group. Moreover, no significant differences were seen between RF1 and RF2 samples from the single fasting volunteer, or among the fasting group in general.

Table 4.7 *P*-values are represented to show the significant test for creatinine, arsenic species and total arsenic after creatinine adjustment.

	***P*-value**					
Sample group and number (n)	creatinine	AB	DMA	MA	Sum of species	Total arsenic
Single fasting volunteer						
RF1(5) vs RF2 (5)	0.07	0.50 0.33[a]	0.53 0.33[a]	N/A	0.70	0.81
Fasting group						
RF1(29) vs RF2 (29)	0.13	0.35 0.30[a]	0.94 0.30[a]	0.51 0.15[a]	0.55	0.85
Fasting group (RF) and non-fasting group (NF)[b]						
RF(29) vs non-fasting group (63)	0.07	0.46 0.58[a]	0.05 0.80[a]	0.00 0.01[a]	0.88	0.75

[a] These *P*-values are associated with species (AB, DMA and MA) percentages.

[b] non-fasting group correspond to data for the three ethnic groups (Asian, Somali and White) taken from Chapter 3.

4.4 Discussion

The present investigation is the first of its kind to evaluate the effect of Ramadan fasting on urinary arsenic levels of unexposed volunteers practising Ramadan fasting. A Ramadan fasting (RF) group was recruited from the same city (Leicester, UK), who were not exposed to arsenic-contaminated water. The volunteers were asked to refrain from fish and seafood consumption for three days prior to urine collection.

The vast majority of the volunteers have a very similar arsenic concentrations in their urine samples (Fig. 4.5). Only few volunteers showed high levels of arsenic in their urine samples. The precise reason for this is not clear. It could be due to the volunteers not strictly adhering to the instructions given with the questionnaire, especially regarding the need to avoid consumption of seafood for 3 days prior to sample collection. As can be seen from Fig. 4.5, creatinine adjustment results in a reduction in the number of outliers. The reduction in number of outliers after creatinine adjustment indicates the importance of creatinine adjustment. Creatinine adjustment eliminates the role of different factors that are not related to arsenic exposure, such as urine volume and concentration, which is widely used (Hinwood et al., 2002; Suwazono et al., 2005) and Vahter et al., 2006).

Analysing of the data for 29 volunteers revealed only few outliers. The exclusion or inclusion of these values in the analysis did not influence the significance of the findings. The results of this study were compared with the results of non-fasting group (ethnic group: Asian, Somali and White) from Chapter 3. No significant difference ($P > 0.05$) in both total arsenic and arsenic species for the two different groups was found. This indicates that Ramadan fasting has no effect on total and the sum of urinary arsenic species. Moreover, by comparing the two different samples types (RF1 and RF2) of fasting group, there was no significant difference ($P > 0.05$) in both the total and arsenic species was found. This further suggests that during the course of a Ramadan fasting there is no significant effect on urinary arsenic with regard to the total levels excreted at the beginning and the end of a 12h fast. Similarly, there was also no significant difference between the urine samples, for the single fasting

volunteer, at these two time points. However, the pattern of urinary arsenic species showed difference between fasting and non-fasting groups, and among the fasting group at the beginning and at the end of the fasting period.

Methylarsonate was detected more frequently in the fasting group compared to the non-fasting group, which was 12-fold higher in the fasting than in non-fasting group. In the fasting group, the frequency of detection of MA was 2-fold higher in RF2 than in RF1. This finding may be taken to suggest that the human body, during Ramadan fasting, creates an environment that favours the elimination of the more toxic organoarsenic species (MA). Volunteers who practice Ramadan fasting excrete more of a toxic form of organoarsenic from the body than those who eat at regular intervals. This suggests that the way arsenic is excreted from the body changes during periods of fasting

One could argue that daytime activity rather than fasting *per se* may be the reason for this. Daytime activity is a possible confounding factor that could not be eliminated. However, a recent study comparing spot urine with 24 hour urine revealed that no significant differences was observed in urinary arsenic species between these two different points of time collection (Hinwood et al., 2002), which suggests that daytime activity does not influence urinary arsenic species distribution.

The reason for the increase in MA percentages and a decrease in AB percentages in urine samples collected at sunset (RF2), after approximately 12 hours fasting, is unlikely to be due to diet since no food was consumed between the two time points of the samples collection. The finding also highlights the need for caution in use of MA as a biomarker for arsenic exposure, since it can be detected at higher

percentage due to fasting condition. Furthermore, this finding indicates that the time point of urine sample collection and the nutritional status (fasting/starvation) of the individual should be known.

Generally, urinary arsenic analysis involves collection of first morning void urine. Previously it has been reported that total arsenic level and species does not significantly vary between spot urine samples and that collected over a 24 hours period (Hinwood et al., 2002). However, that study did not investigate the situation where the body is subjected to a condition where it is deprived from the consumption of food and fluid for over 12 hours during the daytime.

One possible explanation for the increase in the percentage of MA in Ramadan fasting urine samples (RF2; collected at sunset before the breaking of the fast) could be related to the biomethylation process, attributed to the altered metabolic state of the body caused by the fasting. It has been previously reported that Ramadan fasting results in a reduction of homocysteine levels (Aksungar et al., 2005). Homocysteine is considered as a factor that influences arsenic methylation. Therefore, it can be suggested that a lowering of the homocysteine level will result in an increase in the percentage of DMA and a significant increase in MA. An elevated level of homocysteine was reported to negatively influence the biosynthesis of S-adenosylmethionine and glutathione (Alan et al., 1997). These latter compounds were well known as being essential for arsenic biotransformation in humans. Arsenic methylation takes place in the liver where the arsenic receives a methyl group from S-adenosylmethionine in its trivalent oxidation state, in which reduced glutathione is required (Vahter, 1994). Since homocysteine is a substrate for methionine, the

reduction of homocysteine levels would increase the availability of methionine for the biomethylation of arsenic. This is because homocysteine is normally metabolised through two biochemical pathways: re-methylation and trans-sulfuration. Homocysteine is converted to methionine via the first pathway and to cysteine and taurine through the latter pathway (Miller, 2003).

The methylation process of arsenic is a biochemical pathway that is also dependent on folate (Gamble et al., 2005), which has been reported to increase under fasting condition (Cahill et al., 1998). This could also offer an explanation for the observed distribution of arsenic species during the two time points of Ramadan fasting investigated in this study. DMA is positively associated with plasma folate (Gamble et al., 2005). The increased level of folate in animals was also reported to increase biomethylation processes (Spiegelstein et al., 2003). Thus, one can postulate that during the course of fasting, the level of folate increases, which results in an increase in the percentage of DMA and a significant increase of detected MA among RF2 urine samples.

It is noteworthy that there was a high percentage of AB in almost all the urine samples of the two types with average percentages of 64% AB in RF1 and 60% AB in RF2, although all the volunteers were asked to refrain from eating fish and seafood for three days prior to sample collection. There was no significant ($P > 0.05$) difference between RF1 and RF2 regarding the level of AB. It is worth mentioning that previous studies have also reported high levels of AB in urine (Ritsma et al., 1998; Lai et al., 2004). For example, Ritsma et al. (1998) found 69.5% AB in the urine of healthy volunteers who refrained from seafood consumption for two days. An explanation for

this may be linked to other sources of AB in the diet. For example, it has recently been reported that AB is present in chicken (Polatajko et al., 2004) which is widely consumed among the volunteers used in this study based on the data from a questionnaire (Chapter 3). Furthermore, Consumption of fish ingredients in some products cannot be ruled out (Ritsema et al., 1998).

4.5 Conclusion

The data from this study were analysed for each individual within different groups. Only few individuals showed major deviation from the rest of the population within each group. However, inclusion or exclusion of these values does not alter the statistical significance of the outcome. The more frequent detection of MA seen in the fasting group, compared with the non-fasting group (data taken from Chapter 3), and at the end of the fast compared to the beginning of the fast, provides support for an effect of fasting on urinary arsenic species distribution. In addition, the increase in the percentage of DMA and MA detected at the end of an approximately 12h long fast, compared to the beginning of the fast, suggests that the human body, during the fasting period favours the removal of the most toxic methylated species detected in this study. However, fasting has no significant effect on the total level of urinary arsenic seen at the start and after the 12 hours long fast. Further study on a larger group of volunteers and different types of fasting is required to confirm the finding of this research especially in epidemiological areas, where the people are exposed to high arsenic concentration through drinking water.

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Chapter 5

Urinary Arsenic and Selenium Ratio

This Chapter describes the determination of the baseline value for urinary arsenic (As): selenium (Se) ratio for an unexposed population in Leicester, United Kingdom.

5.1 Introduction

The chemical properties and metabolic fate are similar for arsenic and selenium, because both elements are metalloids. The metabolism and toxicity of these two elements have been reported to be modulated by each other (Styblo and Thomas, 2001; Hsieh and Ganther, 1975). Glutathione (GSH) and S-adenosylmethionine (SAM) are necessary for their reductive metabolism and methylation, respectively (Styblo and Thomas, 2001). The metabolism and toxicity of inorganic and organic selenium compounds have been shown to be modified by inorganic arsenic (Hsieh and Ganther, 1975). Selenite (Se (IV)) has been demonstrated to modify the toxicity and metabolism of As (III) (Styblo and Thomas, 2001). Seleno-bis(*S*-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ was identified as a new arsenic-selenium compound in the bile of rabbits injected with aqueous Se (IV) and As (III) solutions (Gailer et al., 2000). This compound contains equimolar arsenic and selenium and provides understanding of the metabolism of Se (IV) and As (III) by animals (Gailer et al., 2000). Although selenium and arsenic have some similarities, they have very different impacts on the human

body. Thus, selenium is considered as an essential micronutrient (NRC, 1989), whereas arsenic is classified as a carcinogen and toxic to humans (IARC, 1987; ATSDR, 2000).

The arsenic and selenium metabolites that are excreted in human urine are discussed in the following paragraphs. In arsenic-exposed populations, arsenic excreted in human urine generally comprises 10 – 30% inorganic arsenic, 10 – 20% MA and 60 – 80% DMA (Hsueh et al., 1997; Hopenhayn-Rich et al., 1996). Urine of unexposed population contains an average percentage of 70% AB, 29% DMA and 1% MA (Chapter 3). The normal level of arsenic in urine has been reported to be in the range of 5 – 40 µg/1.5L/day (Rahman et al., 2005; Farmer and Johnson 1990). In addition, the total concentration of arsenic in urine among unexposed populations in Europe is in the range of 10 -20 µg/l (Ritsema et al, 1998; Stoeppler and Vahter, 1994; Cornelis et al., 1995).

Most of the selenium consumed by humans is excreted in urine. Thus, the amount of selenium detected in urine is a reflection of the dietary intake. After stepwise methylation, selenium is excreted in urine as monomethylated selenium (MSe) and trimethylelenium (TMSe), while dimethylselenide (DMSe) is exhaled in the breath (McConnell and Portman, 1952). In normal human urine Se (IV) and two selenosugars (methyl 2-acetamido-2-deoxy-1-seleno-d-galactopyranoside and methyl 2-amino-2-deoxy-1-seleno-d-galactopyranoside) are considered as typical urinary species (Francesconi et al., 2004). Selenosugar (Se-methyl-N-acetylselenohexosamine) was identified as a major metabolite in human urine (Gammelgaard et al., 2003), after volunteers were exposed to a diet supplemented with selenium. Exposure to 2 µg Se/g diet or drink containing selenium with a concentration 2 µg Se/ml drink, this will

increase arsenosugar level to some extent, while TMSe starts to increase in rats. Therefore, TMSe can be used as a biomarker of excessive selenium dose (Suzuki, 2005b). However, several selenium species remained unidentified. Background levels of selenium in human urine has been reported to be in the range of 10 – 100 µg/l (Wang et al., 2001; Tiez, 1983; Alaejos and Romero, 1993). Various foods are considered as dietary sources of selenium, such as Brazil nuts, grain, wheat, cucumber, mushroom, crab, liver and shellfish (Ogra et al., 2004; Rayman, 2002). Deficiency in selenium intake results in different diseases such as Keshan's disease, numbness in the legs and arms, brittle hair and deformed nails (ATSDR, 2001), while excessive selenium in the diet causes toxic effects such as neruromuscular symptoms and skin lesions (ATSDR, 1996).

Selenium has been reported to have a positive role against arsenic toxicity, because the two elements act as metabolic antipodes (Schrauzer, 1992). Urinary selenium was reported to be associated with an increased proportion of DMA and a decreased inorganic arsenic content in human urine (Christian et al., 2006). It has been reported that selenium and arsenic counteract the toxicity of each other by a mechanism involving reaction of the two elements to form a conjugate in the liver, which is then excreted in the bile (Levander, 1977). Kenyon et al. (1997) carried out a study on mouse to investigate whether differing dietary selenium status could alter urinary arsenic after exposure to As (V). They concluded that an excess of selenium in the diet was associated with excretion of high proportion of urinary inorganic arsenic and low proportion of organic arsenic, respectively, compared with a selenium sufficient diet.

In contrast, a selenium deficient diet was found to eliminate As (V), As (III) and DMA in urine more slowly than a selenium sufficient diet.

In another study with humans, selenium was shown to be an effective treatment against arsenism, a disease that result from long-term exposure to high arsenic in the environment (Wuyi et al., 2001). After administration of 100 - 200 µg Se/day to patients and control groups for 14 months, the patients showed decreased levels of arsenic in blood, hair and urine compared to those of the control group (Wuyi et al., 2001). This would suggest that for health risk assessment among populations exposed to arsenic, selenium measurement is also important to estimate the extent of arsenic toxicity.

Whilst some studies have been reported on the relationship between arsenic and selenium, they have been limited to studies of arsenic exposed populations (Hsueh et al., 2003; Christain et al., 2006). The study with exposed populations demonstrated a positive correlation between arsenic and selenium in urine and hair (Hsueh et al., 2003; Spallholz et al., 2005) although one study (Miyazaki et al., 2003) suggested that there was negative correlation between both elements.

Results presented in this Chapter is based on study of unexposed volunteers from different ethnic groups, fasting volunteers and a single volunteer whose urinary arsenic and selenium levels were monitored over a period of one year. The objective of the study was to establish a baseline level of urinary As:Se ratio, in addition to further understanding the relationship between arsenic and selenium in unexposed populations.

5.2 Experimental

This section covers the following sub-sections: chemical reagents, instrumentation, samples collection, quality control, creatinine determination and statistical analysis.

5.2.1 Chemicals and reagents

The chemicals reagents used for experiments related to this Chapter are same as what has been reported in Chapter 3, section 3.2.2, section, with the exception of the following: a selenium stock solution standard was purchased from Sigma-Aldrich (Germany). Selenium atomic absorption standard solution contains 978 µg/ml of Se in 1.4 wt. % HNO_3.

5.2.2 Instrumentation

Determination of total arsenic and selenium in urine was performed by monitoring signal at m/z = 75 and 82. Interference on ^{75}As by $^{40}Ar^{35}Cl$ was corrected by monitoring the ion counts at m/z = 77, 82 and 83 and applying the correction equation pre-programmed in the ICP-MS software (Elan Instrument Control, version 3.0 hotfix 3). Possible interference from $^{40}Ar^{35}Cl$ was overcome by chromatographic resolution of chloride from all five arsenic species (AB, DMA, MA, As (III) and As (V)). Analysis of both totals (arsenic and selenium) and arsenic species were carried out as what has been reported in Chapter 2: sections 2.2.4 and 2.2.5, respectively.

5.2.3 Samples collection and preparation

Urine sample collection and storage were carried out as reported in Chapter 3, section 3.2.1.2 and Chapter 4, section 4.2.3. In addition twelve urine samples were collected from one volunteer over the course of a year without (volunteer part A, n = 6) and with (volunteer part B, n = 6) seafood consumption. In total 133 urine samples were collected from an ethnic group (n = 63, Chapter 3), a fasting group (n = 58, Chapter 4) and one volunteer over one year (n = 12).

5.2.4 Quality Control

A certified reference material (CRM) was used to validate arsenic speciation analysis. The CRM used was human urine No.18 from the National Institute of Environmental Studies (NIES) Japan. The CRM NIES No.18 was reconstituted as described by the manufacturer. Total arsenic and selenium analysis were validated by a spiking experiment, which was also used for arsenic speciation. The spiking experiment for both total arsenic and selenium, and arsenic species in urine, was carried out and the results indicated that any interference present in the urine samples had no effect on the accuracy of the total arsenic or selenium measurements, or the arsenic speciation analysis. The spiking experiment was carried out by using 50 µg/l of each element in urine sample, then diluted (5-fold) with 2% v/v HNO_3 to achieve 10 µg/l total spiked concentration.

5.2.5 Determination of creatinine

Creatinine was analysed as described in Chapter 2, section 2.2.1.3.

5.2.6 Statistical analysis

Statistical test in this Chapter was as reported in Chapter 3, section 3.2.11, and Chapter 4, section 4.2.6. Correlations coefficients and *P*-values were calculated by using statistic software (Wessa, 2007).

5.3. Results

The pH of all urine samples studied was within the range of 5 to 8. This is within the normal range (4.5 – 8.0) expected for the human urine previously reported by Chen et al. (2002). The results of total arsenic and arsenic species, especially for ethnic and fasting group, are reported here for comparative analysis with selenium results in order to determine the As: Se ratio and the relationship between arsenic and selenium in human urine.

5.3.1 Dates of total arsenic and selenium measurements

Dates of collection and dates of measurements and locations for total arsenic and selenium are same as presented in Chapter 3 (ethnic groups, Appendices 3.4, 3.5 and 3.6) and Chapter 4 (fasting group, Appendix 4.3). Total selenium was measured on the same dates and in the laboratory as indicated in these Appendices. Total arsenic and selenium analysis in urine samples were carried out as described in Chapter 2,

section 2.2.4.3 and 2.2.4.4 respectively. Dates of collection and analysis of urine samples of a single volunteer, who monitored for one year, are presented in Appendix 5.1.

5.3.2 Concentration of total arsenic and selenium in urine samples of all volunteers

The concentrations of total arsenic and selenium in the urine samples of all volunteers are given below. The concentrations of both elements were expressed with and without creatinine adjustment among the different groups.

The levels of arsenic, selenium and As:Se ratios for the one-year study of a single volunteer, the ethnic groups (Asian, Somali and White) and the fasting group (paired samples: RF1 and RF2) are shown in Table 5.1 and 5.2 for the single volunteer, Table 5.3, 5.4 and 5.5 for Asian, Somali and Whites, respectively. Table 5.6 and 5.7 presents the data for the fasting group, for RF1 and RF2, respectively. All these Tables show total arsenic and selenium levels before and after creatinine adjustment.

Table 5.1 Single volunteer: concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Seafood ingestion days ago	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg As/g creatinine)	As:Se ratio
1.	Au04U	2	65.6	21.3	2590		25.3	8.2	3.1
2.	Sep04U	7	31.8	25.1	2420		13.1	10.4	1.3
3.	Oct04U	7	27.8	20.5	2036		13.7	10.1	1.4
4.	Nov04U	2	22.5	7.5	961		23.4	7.8	3.0
5.	Dec04U	2	47.0	17.1	2556		18.4	6.7	2.7
6.	Jan05U	1	119.8	16.1	1267		94.6	12.7	7.4
7.	Feb05U	1	115.7	11.4	2409		48.0	4.7	10.1
8.	Mar05U	1	66.3	23.7	2613		25.4	9.1	2.8
9.	Apr05U	14	15.9	22.0	2726		5.8	8.1	0.7
10.	May05U	14	12.9	19.6	1759		7.3	11.1	0.7
11.	Jun05U	7	12.8	18.6	2785		4.6	6.7	0.7
12.	Jul05U	21	38.8	29.9	3370		11.5	8.9	1.3
	Mean		**48.1**	**19.4**	**2291**		**24.3**	**8.7**	**2.9**
	SD		**37.3**	**6.0**	**678**		**25.1**	**2.2**	**2.9**
	Median		**35.3**	**20.1**	**2488**		**16.0**	**8.5**	**2.1**
	Min.		**12.8**	**7.5**	**961**		**4.6**	**4.7**	**0.7**
	Max.		**119.8**	**29.9**	**3370**		**94.6**	**12.7**	**10.1**

Data from Table 5.1 are divided into two parts depending on whether seafood was ingested shortly before urine sample collection or not. Volunteer part A (no recent seafood > 7 days ago), Volunteer part B (recent seafood < 3 days ago). These are detailed in Table 5.2.

Table 5.2 For the one year study single volunteer split into two sample groups; refraining from or ingesting seafood.

As (µg/g creatinine)	Samples (n)	Mean	SD	Median	Min.	Max.
	Volunteer part A* (6)	11.6	3.5	13.2	4.6	13.7
	Volunteer part B* (6)	39.2	29.0	25.4	18.4	94.6
Se(µg/g creatinine)						
	Volunteer part A (6)	9.2	1.7	9.5	6.7	11.2
	Volunteer part B (6)	8.2	2.7	8.0	4.7	12.7
As: Se						
	Volunteer part A (6)	1.2	0.3	1.3	0.7	1.6
	Volunteer part B (6)	4.9	3.2	3.0	2.7	10.1

*Volunteer part A (no recent seafood ingestion) and Volunteer part B (recent seafood ingestion).

Table 5.3 Asian group: concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg As/g creatinine)	As:Se ratio
1.	UA1	30.5	29.5	1868		16.3	15.8	1.0
2.	UA2	17.9	38.9	3136		5.7	12.4	0.5
3.	UA3	70.5	60.2	1709		41.3	35.2	1.2
4.	UA4	55.2	36.3	1823		30.3	19.9	1.5
5.	UA5	60.4	81.9	2139		28.2	38.3	0.7
6.	UA6	47.3	83.1	2151		22.0	38.6	0.6
7.	UA7	198.8	108.5	2355		84.4	46.1	1.8
8.	UA8	17.4	27.7	1347		12.9	20.5	0.6
9.	UA9	11.8	24.2	1166		10.1	20.7	0.5
10.	UA10	15.6	68.8	2332		6.7	29.5	0.2
11.	UA11	40.7	38.6	1902		21.4	20.3	1.1
12.	UA12	44.5	39.6	2264		19.7	17.5	1.1
13.	UA13	25.2	25.6	1641		15.4	15.6	1.0
14.	UA14	21.9	45.3	1562		14.0	29.0	0.5
15.	UA15	18.6	38.6	2660		7.0	14.5	0.5
16.	UA16	8.6	25.5	1075		8.0	23.7	0.3
17.	UA17	16.5	42.8	1404		11.8	30.5	0.4
18.	UA18	63.8	55.1	1958		32.6	28.2	1.2
19.	UA19	6.5	7.8	283		23.0	27.5	0.8
20.	UA20	10.1	27.6	1404		7.2	19.6	0.4
21.	UA21	33.8	57.2	2434		13.9	23.5	0.6
	Mean	**39.0**	**45.8**	**1838.7**		**20.6**	**25.1**	**0.8**
	SD	**41.0**	**24.0**	**625.2**		**15.4**	**9.0**	**0.4**
	Median	**25.2**	**38.9**	**1868.0**		**17.5**	**23.5**	**0.6**
	Min.	**6.5**	**7.8**	**283.0**		**5.7**	**12.4**	**0.2**
	Max.	**198.8**	**108.5**	**3136.0**		**84.4**	**46.1**	**1.8**

Table 5.4 Somali group: concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg As/g creatinine)	As:Se ratio
1.	US1	8.1	23.6	1030		7.9	23.0	0.3
2.	US2	4.6	15.3	758		6.1	20.2	0.3
3.	US3	3.1	14.0	826		3.8	16.9	0.2
4.	US4	12.2	39.4	1664		7.3	23.7	0.3
5.	US5	22.6	32.9	1811		12.5	18.2	0.7
6.	US6	10.2	17.4	838		12.2	20.8	0.6
7.	US7	7.5	19.2	1087		6.9	17.6	0.4
8.	US8	13.2	37.1	1290		10.2	28.8	0.4
9.	US9	10.7	18.6	724		14.8	25.6	0.6
10.	US10	4.5	9.2	634		7.1	14.6	0.5
11.	US11	7.7	13.4	577		13.3	23.2	0.6
12.	US12	3.2	13.5	634		5.0	21.3	0.2
13.	US13	3.4	12.0	577		5.9	20.7	0.3
14.	US14	3.3	6.0	838		3.9	7.2	0.5
15.	US15	6.2	10.4	1087		5.7	9.6	0.6
16.	US16	7.8	110.2	906		8.6	121.6	0.1
17.	US17	8.8	16.6	1449		6.1	11.4	0.5
18.	US18	7.8	30.6	3804		2.1	8.1	0.3
19.	US19	2.0	3.4	5060		0.4	0.7	0.6
20.	US20	12.5	34.9	2422		5.2	14.4	0.4
21.	US21	4.6	29.9	1336		3.4	22.3	0.2
22.	US22	18.6	33.1	1743		10.7	19.0	0.6
	Mean	**8.3**	**24.6**	**1413.4**		**7.2**	**22.2**	**0.4**
	SD	**5.2**	**21.9**	**1102.5**		**3.8**	**23.2**	**0.2**
	Median	**7.8**	**18.0**	**1058.5**		**6.5**	**19.6**	**0.4**
	Min.	**2.0**	**3.4**	**577**		**0.4**	**0.7**	**0.1**
	Max.	**22.6**	**110.02**	**5060**		**14.8**	**121.6**	**0.7**

Table 5.5 White group: concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg As/g creatinine)	As:Se ratio
1.	UW1	28.6	63.3	1472		19.4	43.0	0.5
2.	UW2	9.4	20.0	374		25.1	53.4	0.5
3.	UW3	5.2	19.9	464		11.2	42.9	0.3
4.	UW4	56.7	44.7	1698		33.4	26.3	1.3
5.	UW5	30.1	42.3	2332		12.9	18.1	0.7
6.	UW6	61.7	42.6	940		65.6	45.3	1.4
7.	UW7	16.2	25.9	770		21.0	33.6	0.6
8.	UW8	9.5	30.1	996		9.5	30.2	0.3
9.	UW9	5.7	11.2	351		16.2	31.9	0.5
10.	UW10	23.3	62.9	1324		17.6	47.5	0.4
11.	UW11	11.9	37.5	679		17.5	55.3	0.3
12.	UW12	27.1	18.5	543		49.9	34.1	1.5
13.	UW13	24.2	16.3	340		71.2	48.0	1.5
14.	UW14	7.9	29.0	917		8.6	31.6	0.3
15.	UW15	5.4	10.1	159		34.0	63.7	0.5
16.	UW16	8.3	21.7	1053		7.9	20.6	0.4
17.	UW17	10.1	24.0	951		10.6	25.2	0.4
18.	UW18	17.9	17.0	419		42.7	40.6	1.1
19.	UW19	9.1	22.0	1426		6.4	15.5	0.4
20.	UW20	4.2	12.1	475		8.8	25.5	0.3
	Mean	**18.6**	**28.5**	**884.2**		**24.5**	**36.6**	**0.7**
	SD	**16.2**	**15.7**	**549.6**		**19.3**	**13.2**	**0.4**
	Median	**11.0**	**23.0**	**843.5**		**17.6**	**33.9**	**0.5**
	Min.	**4.2**	**10.1**	**159**		**6.4**	**15.5**	**0.3**
	Max.	**61.7**	**63.3**	**2332**		**71.2**	**63.7**	**1.5**

Table 5.6 Fasting group (RF1*): concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg Se/g creatinine)	As:Se ratio
1.	UNF1	5.3	6.3	804		6.5	7.8	0.8
2.	UNF2	20.2	58.9	3090		6.6	19.1	0.3
3.	UNF3	33.8	38.8	2196		15.4	17.7	0.9
4.	UNF4	12.2	29.2	1472		8.3	19.8	0.4
5.	UNF5	16.4	48.9	2660		6.1	18.4	0.3
6.	UNF6	83.6	66.2	3396		24.6	19.5	1.3
7.	UNF7	14.2	15.9	1211		11.7	13.2	0.9
8.	UNF8	12.2	29.5	1234		9.9	23.9	0.4
9.	UNF9	30.9	48.4	3328		9.3	14.5	0.6
10.	UNF10	25.2	27.3	1189		21.2	23.0	0.9
11.	UNF12	25.7	34.6	985		26.1	35.2	0.7
12.	UNF13	16.3	24.7	1223		13.4	20.2	0.7
13.	UNF14	12.7	24.1	1041		12.2	23.1	0.5
14.	UNF15	16.1	25.6	532		30.2	48.1	0.6
15.	UNF16	78.9	54.7	1845		42.8	29.6	1.4
16.	UNF17	51.0	45.5	1245		41	36.5	1.1
17.	UNF19	7.8	30.1	1381		5.7	21.8	0.3
18.	UNF21	9.2	17.6	589		15.7	29.9	0.5
19.	UNF22	10.1	21.9	951		10.6	23.1	0.5
20.	UNF24	15.5	16.4	894		17.3	18.3	0.9
21.	UNF25	15.5	65.2	2287		6.8	28.5	0.2
22.	UNF26	13.1	14.7	238		54.9	61.8	0.9
23.	UNF27	10.5	16.0	724		14.5	22.1	0.7
24.	UNF28	9.1	19.8	498		18.3	39.8	0.5
25.	UNF29	37.1	35.9	2004		18.5	17.9	1.0
26.	UNF30	13.0	32.1	679		19.2	47.3	0.4
27.	UNF32	46.0	40.5	2015		22.8	20.1	1.1
28.	UNF33	42.3	66.7	2117		20	31.5	0.6
29.	UNF34	38.3	47.8	1789		21.4	26.7	0.8
	Mean	**24.9**	**34.6**	**1504**		**18.3**	**26.2**	**0.7**
	SD	**19.9**	**16.9**	**859.7**		**11.8**	**11.7**	**0.3**
	Median	**16.1**	**30.1**	**1234**		**15.7**	**23.0**	**0.7**
	Min.	**5.3**	**6.3**	**238**		**5.7**	**7.8**	**0.2**
	Max.	**83.6**	**66.7**	**3396**		**54.9**	**61.8**	**1.4**

*RF1 and RF2 are paired urine samples, collected from the same person at the beginning and at the end of the fasting period, respectively

Table 5.7 Fasting group (RF2*): concentrations of total arsenic and selenium in urine samples before and after creatinine adjustment and As:Se ratio.

Sample No.	Sample name	Total arsenic (µg/l)	Total selenium (µg/l)	Creatinine (mg/l)		Total arsenic (µg As/g creatinine)	Total selenium (µg Se/g creatinine)	As:Se ratio
1.	UF1	64.4	64.7	4403.0		14.6	14.7	1.0
2.	UF2	20.1	53.2	3022.0		6.7	17.6	0.4
3.	UF3	27.8	58.9	4030.0		6.9	14.6	0.5
4.	UF4	25.1	46.2	2106.0		11.9	21.9	0.5
5.	UF5	7.6	28.2	985.0		7.7	28.6	0.3
6.	UF6	136.8	40.3	2049.0		66.8	19.7	3.4
7.	UF7	33.8	34.2	1709.0		19.8	20.0	1.0
8.	UF8	35.3	90.5	3509.0		10.0	25.8	0.4
9.	UF9	26.0	39.5	1936.0		13.4	20.4	0.7
10.	UF10	9.1	40.1	1460.0		6.3	27.4	0.2
11.	UF12	27.1	37.2	860.0		31.5	43.2	0.7
12.	UF13	18.3	41.4	1211.0		15.2	34.2	0.4
13.	UF14	22.3	41.9	951.0		23.4	44.1	0.5
14.	UF15	33.1	55.1	1743.0		19.0	31.6	0.6
15.	UF16	21.2	45.9	2083.0		10.2	22.0	0.5
16.	UF17	22.1	77.1	1721.0		12.8	44.8	0.3
17.	UF19	23.2	39.7	1245.0		18.6	31.9	0.6
18.	UF21	11.5	41.4	1675.0		6.9	24.7	0.3
19.	UF22	7.2	22.7	272.0		26.6	83.4	0.3
20.	UF24	48.0	58.5	1562.0		30.7	37.4	0.8
21.	UF25	12.8	34.5	702.0		18.2	49.1	0.4
22.	UF26	22.5	41.3	1789.0		12.6	23.1	0.5
23.	UF27	7.0	10.4	3384.0		2.1	3.1	0.7
24.	UF28	16.5	48.0	1823.0		9.1	26.3	0.3
25.	UF29	96.1	53.6	2932.0		32.8	18.3	1.8
26.	UF30	19.3	42.7	1664.0		11.6	25.7	0.5
27.	UF32	42.2	53.4	2151.0		19.6	24.8	0.8
28.	UF33	25.3	49.7	951.0		26.6	52.3	0.5
29.	UF34	14.0	27.3	623.0		22.5	43.8	0.5
	Mean	**30.2**	**45.4**	**1881.1**		**17.7**	**30.2**	**0.7**
	SD	**27.6**	**15.8**	**1016.6**		**12.5**	**15.4**	**0.6**
	Median	**22.5**	**41.9**	**1721**		**14.6**	**25.8**	**0.5**
	Min.	**7.0**	**10.4**	**272**		**2.1**	**3.1**	**0.2**
	Max.	**136.8**	**90.5**	**4403**		**66.8**	**83.4**	**3.4**

*RF1 and RF2 are paired urine samples, collected from the same person at the beginning and at the end of the fasting period, respectively.

Outliers for total arsenic have been already discussed in Chapter 3 and 4. It was found that creatinine adjustment resulted in a reduction of outliers. As shown in Fig. 5.1 two groups (White and RF1) appear to have no outliers for selenium before creatinine adjustment. However, there are outliers seen for the other groups before creatinine adjustment and these include the following: Asian (108 µg/l), Somali (110 µg/l), RF2 (10.4, 90.5, and 77.1 µg/l). After creatinine adjustment, Asian and White groups have no outliers (Fig. 5.2). The outliers seen after creatinine adjustment are as follows: Somali (122 µg Se/g creatinine), RF1 (61.8 µg Se/g creatinine) and RF2 (83.4µg Se/g creatinine). The As:Se ratios among all these groups did not show any outliers before and after creatinine adjustment (Fig. 5.3), except for the fasting group (RF2), which had two outliers (1.8 and 3.4).

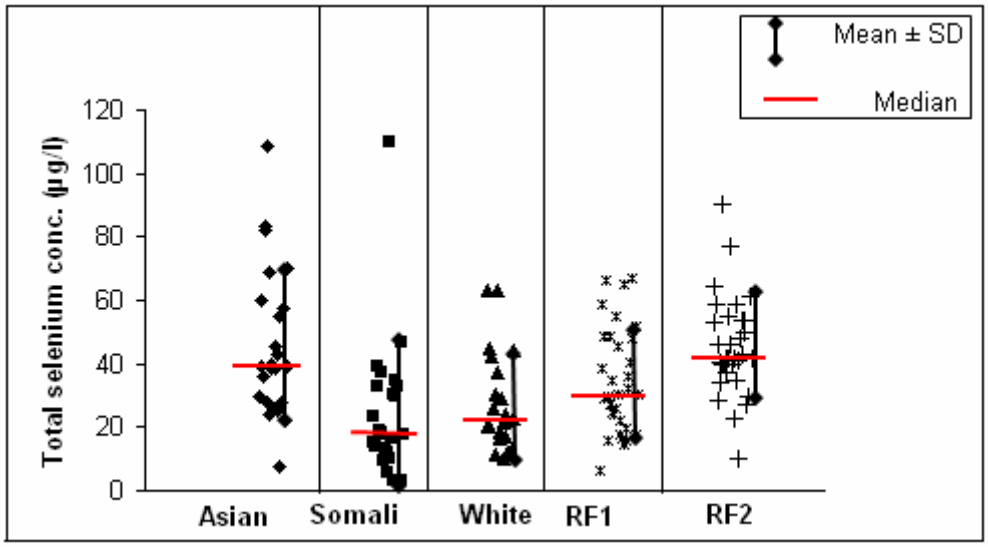

Figure 5.1 Urinary selenium concentration (µg/l) before creatinine adjustment among the three ethnic groups (Asian, Somali and White) and the fasting group (RF1 and RF2).

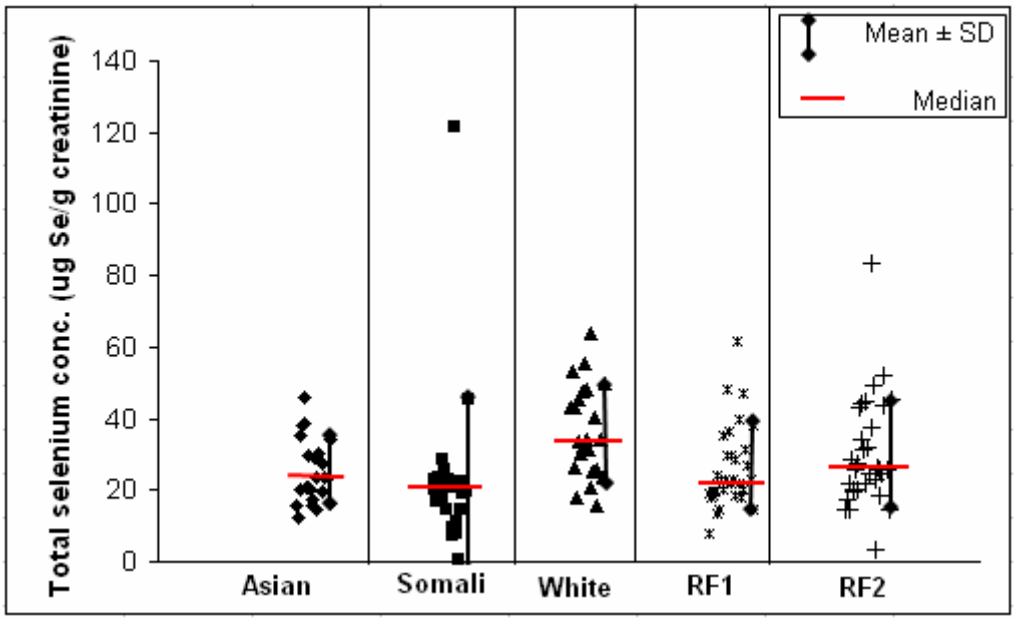

Figure 5.2 Urinary selenium concentration (µg Se/g creatinine) after creatinine adjustment among the three ethnic groups (Asian, Somali and White) and the fasting group (RF1 and RF2).

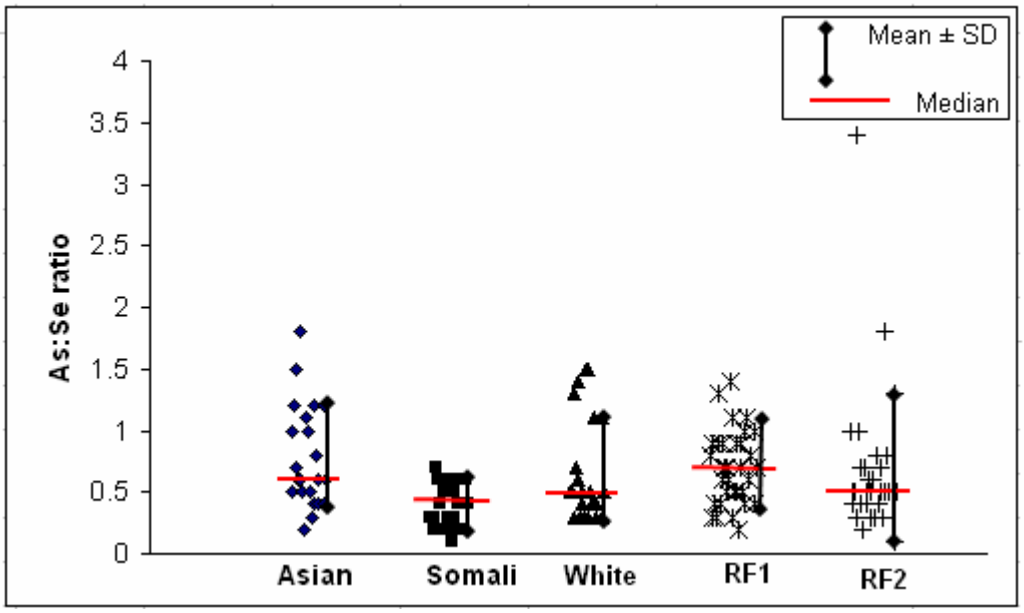

Figure 5.3 As: Se ratio among the three ethnic groups (Asian, Somali and White) and fasting group (RF1 and RF2).

Table 5.8 shows *P*-values to test the influence of seafood consumption (the single volunteer), ethnicity, and fasting on total arsenic, selenium and As:Se ratio. Seafood consumption and ethnicity showed a significant influence ($P < 0.05$) on the As: Se ratio. The results of one volunteer that was monitored for one year for total urinary arsenic and selenium levels, showed the influence of seafood consumption on arsenic level, which has a significant effect on As:Se ratio. This is because selenium levels remained relatively constant and the arsenic level changed due to seafood consumption. The Somali group showed a significant difference ($P < 0.05$) compared with the Asian and White groups, regarding total arsenic level, which was discussed in Chapter 3. The White ethnic group showed a significant difference ($P < 0.05$) in the selenium level compared with the Asian and Somali groups. Fasting showed no significant effect on total arsenic, total selenium and As:Se ratio for the volunteers whose urine was collected before and after an approximately 12h long fast (Table 5.8).

Table 5.8 *P*-values show the significant test for total arsenic, total selenium and As: Se ratio, in urine samples among different groups.

	***P*-value**		
One volunteer	Total arsenic	Total selenium	As:Se ratio
Part A(6) vs. Part B (6)	0.04	0.45	0.02
Ethnic groups			
Asian (21) vs. Somali (22)	0.00	0.60	0.00
Asian (21) vs. White (20)	0.50	0.00	0.35
Somali (22) vs. White (20)	0.00	0.02	0.02
Fasting group			
RF1 (29) vs. RF2(29)	0.85	0.31	0.80

Urine samples were collected from one volunteer over one year, without (volunteer part A, no seafood consumption for more than one week) and with seafood consumption (volunteer part B, seafood consumption within three days). The ratio of As:Se for the volunteer remains constant (part A 1.2 ± 0.3) when refraining from seafood consumption. However, this ratio alters after seafood consumption (part B 4.9 ± 3.2). As shown in Fig.5.4 the clearance of arsenic originating from seafood is achieved 3 days after the ingestion of seafood. It is clear from this graph that seafood ingestion has an effect on both total arsenic levels and the As:Se ratio, but the selenium levels were unaffected.

The As:Se ratios in urine samples for different groups are shown in Fig. 5.5. These are compared with calculated values from the literature studies for unexposed populations from different countries: UK (White et al., 1998), Germany (Heitland and Koster, 2006) and Brazil (Correia et al., 2005).

As:Se ratios were similar for the Asian (0.8 ± 0.4), White (0.7 ± 0.4) and fasting groups (0.7 ± 0.5). The Somali group was significantly different(0.4 ± 0.2). The As:Se ratio in fasting volunteers (0.7 ± 0.5) is similar to the Asian (0.8 ± 0.4) and the White (0.7 ± 0.4) groups, with the exception of Somali group (0.4 ± 0.2.). This suggests that the diurnal fasting does not result in an overall alteration in As:Se ratio, which is also evident from the results for RF1 (0.7 ± 0.3) and RF2 (0.7 ± 0.6).

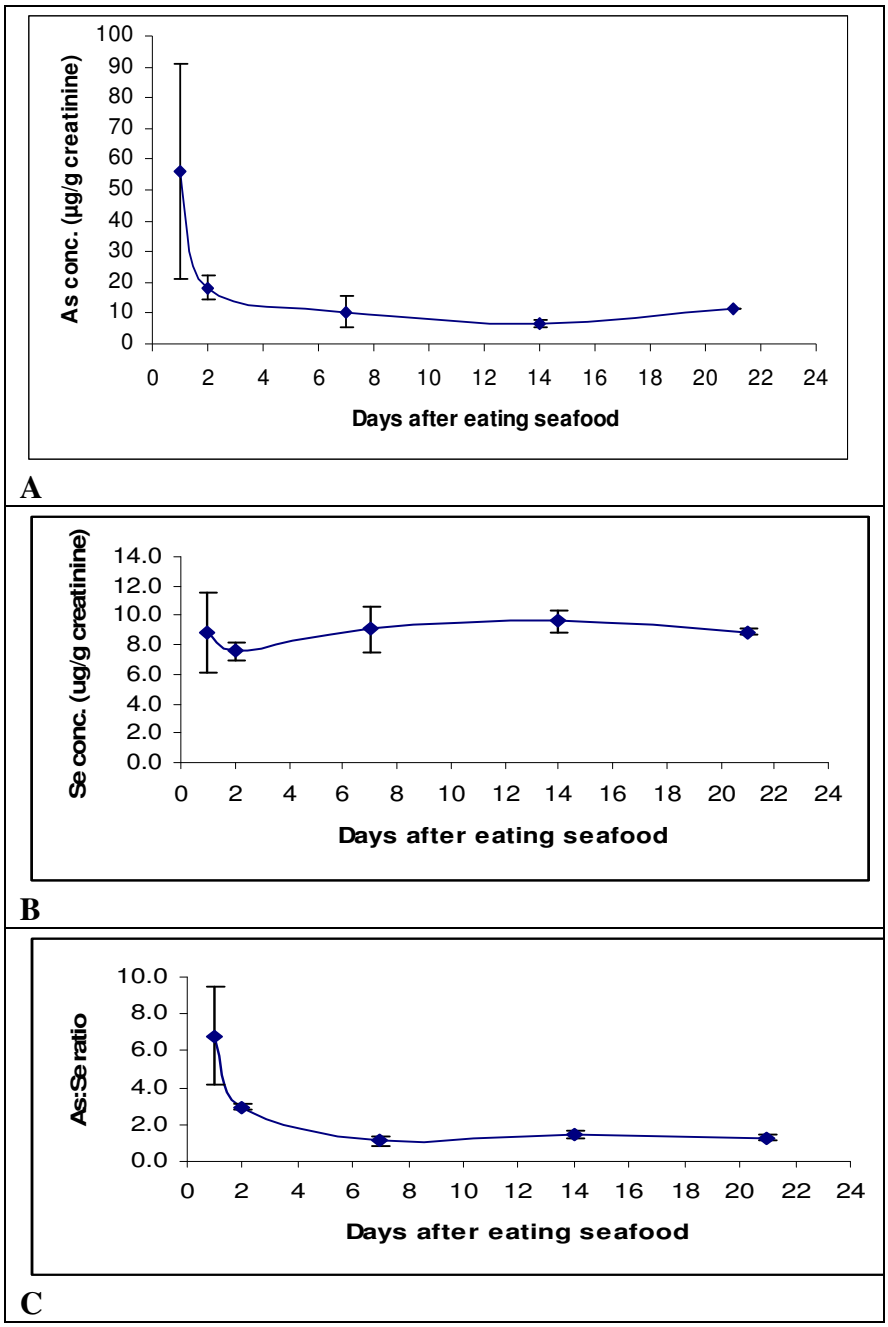

Figure 5.4 Total arsenic (A) , total selenium (B) and As:Se ratio (C) in urine samples of the single volunteer collected over one year and shows the effect of seafood ingestion on the three values; mean values ± SD. The data are presented in Table 5.1.

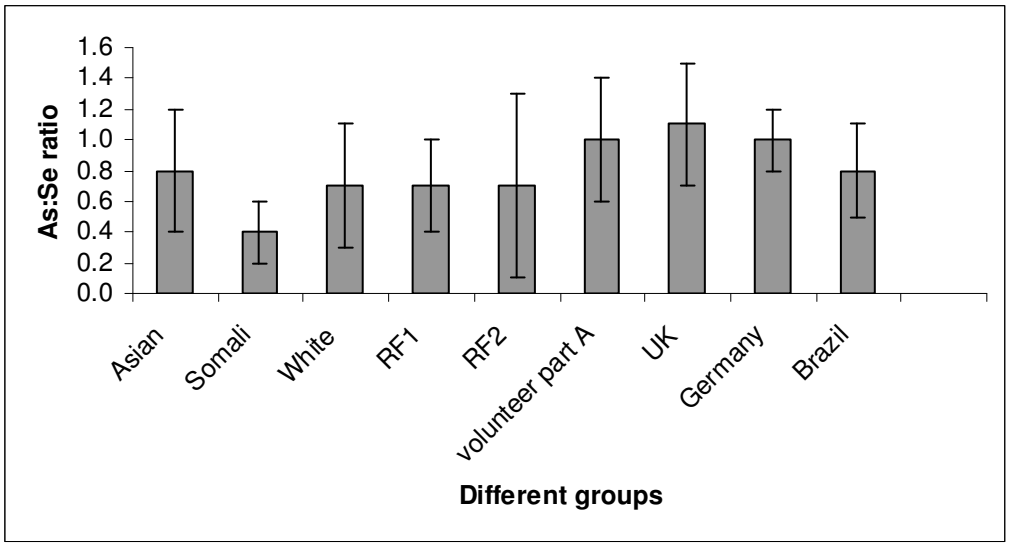

Figure 5.5 As:Se ratios (mean ± SD) in urine samples of unexposed populations in UK: Asian, Somali and White; fasting groups (RF1 and RF2); volunteer part A (without seafood).

The Somali group displays the lowest As:Se ratio, indicating that the level of excreted selenium is higher than that of arsenic , which is true for all the ethnic groups. However, the exceptional situation for the Somali group is that the level of selenium is 3.1-fold higher than that of arsenic, compared to 1.3 and 1.5-fold higher for the Asian and White groups, respectively. The As:Se ratios were also calculated from the literature (Table 5.9) based on studies in different countries: UK (White et al., 1998), Germany (Heitland and Koster, 2006) and Brazil (Correia et al., 2005). Table 5.9 shows the arsenic, selenium, and As:Se values determined in this study together with the ratios calculated from literature reports of arsenic and selenium levels in other

studies. It is evident from this Table that, with the exception of the Somali group, the average As:Se ratio is between 0.7-1.2 for all the studies reported so far, to the best of my knowledge, regarding arsenic and selenium levels in human urine.

Table 5.9 As:Se ratios in urine samples of exposed and unexposed volunteers to high arsenic level in drinking water or seafood.

	No. Urine Samples	Arsenic conc. µg/g creatinine Mean ±SD	Selenium conc. µg/g creatinine Mean ± SD	As:Se (Ratio) Mean ±SD	Reference
Unexposed[§]					
UK(White)	20	24.5 ± 19.0	36.6 ± 13.2	0.7 ± 0.4	This work
UK (Asian)	21	20.6 ± 17.5	25.1 ± 9.0	0.8 ± 0.4	This work
UK (Somali)	22	7.2 ± 3.8	22.2 ± 23.2	0.4 ± 0.2	This work
UK (one volunteer, part A)	6	9.3 ± 3.9	9.2 ± 2.0	1.2 ± 0.3	This work
UK	200	12.3 µg/l	16.2 (9.2)µg/l	0.8 (1.3),1.1± 0.4	White et al., 1998
Germany†	72[a]	10	12	0.8	Heitland and Koster, 2006
Germany†	87[b]	12	11	1.1	Heitland and Koster,

					2006
Brazil#	4	28.5 ± 7.0 µg/l	39 ± 7.4 µg/l	0.8 ± 0.3	Correia et al., 2005
Exposed[§]					
UK(one volunteer, part B[»])	6	37.2 ± 30.6	9.2 ± 3.6	4.0 ± 2.7	This work
Bangladesh[γ]	429	181.4 ± 40.2*	20.1 ± 3.8*	9.6 ± 3.8*	Miyazaki et al., 2003
Taiwan[γ]	252	96.9 ± 7.3µg/l	22.4 ± 0.9 µg/l	4.3	Hsueh et al., 2003
Chile[γ]	93	55.8 ± 41.6 µg/l	28.3 ± 13.9µg/l	2.0	Christian et al., 2006

[§] exposed and unexposed to high arsenic level in drinking water or seafood.

No recent seafood consumption (personal communication). [»] exposed to arsenic through seafood ingestion (≤ 2 days ago). [γ] exposed to arsenic in drinking water. † Geometric mean, seafood ingestion was not reported, total arsenic range (1 - 375µg/l); [a] children, [b] Adults.* The mean and SD were calculated after the data were pooled.

Correlations between total arsenic and total selenium in urine samples of different ethnic groups (Asian, Somali and White) and fasting group are shown in Fig. 5.6. Urinary total arsenic concentration was positively linearly correlated with the urinary total selenium concentration. The correlation coefficients for Asian, Somali, White, fasting group (RF1 and RF2) are in the range of 0.5 – 0.7 with *P*-values < 0.05

(Fig. 5.6). This correlation was established after creatinine adjustment for both arsenic and selenium. However, before creatinine adjustment, the correlation was also seen and there was positive correlation among all the different groups. The correlation coefficients for Asian, Somali, White, fasting group (RF1 and RF2) are in the range of 0.4 – 0.8 with *P*-values < 0.05. This shows that the correlation remains unaffected before and after creatinine adjustment. The correlation coefficients and *P*-values are as follows: Asian (r = 0.8, *P* = 0.00), Somali (r = 0.6, *P* = 0.00), White (r = 0.6, *P* = 0.00), RF1 (r = 0.7, *P* = 0.00) and RF2 (r = 0.7 , *P* = 0.00). The correlation coefficient (r) is used to measure the linear relationship between the two data sets. A value of r close to 0 indicates little correlation between two data sets, while r values close to +1 or -1 shows a high level of correlation. In addition, a small *P*-value (< 0.05) is indicative of a correlation.

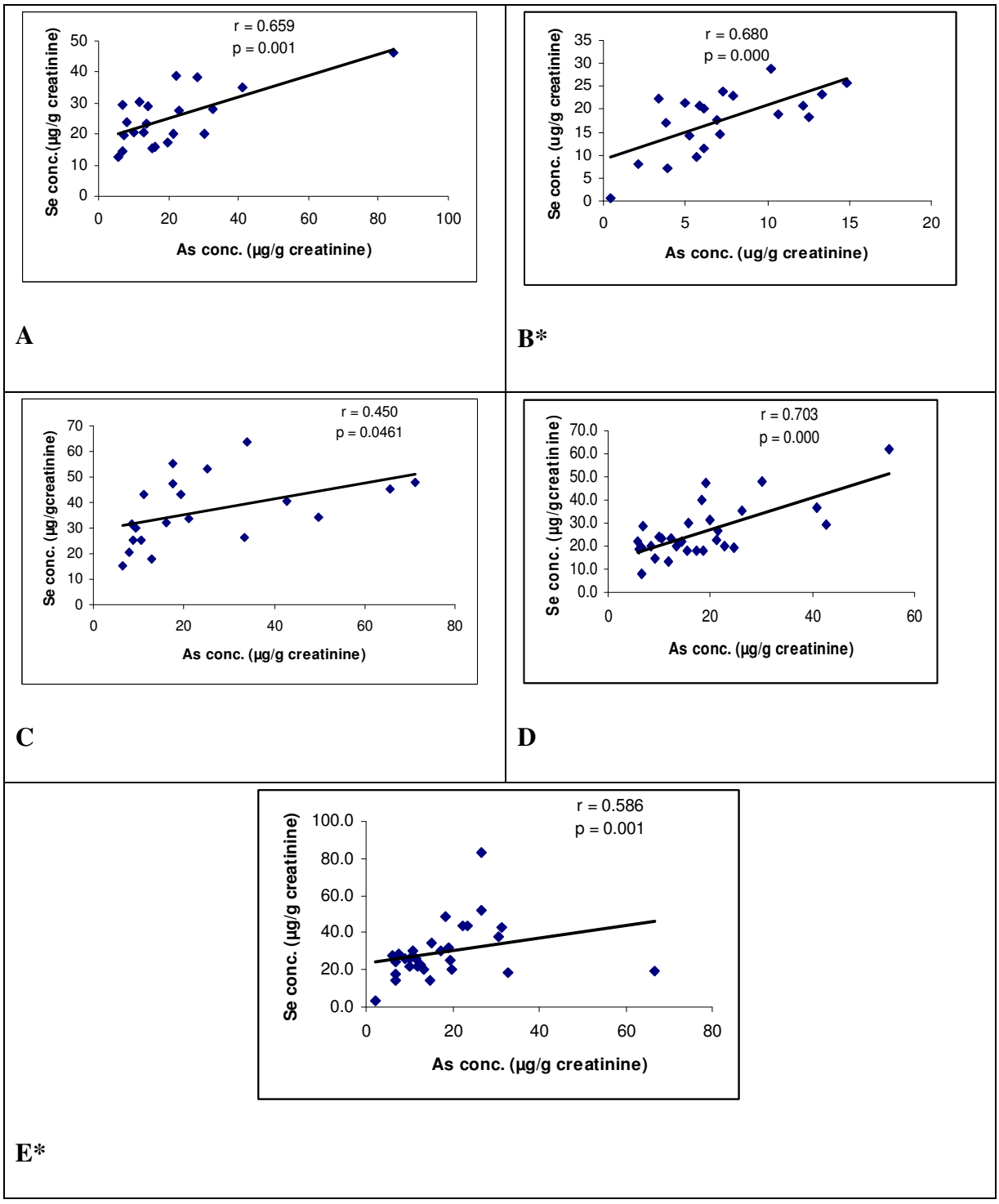

Figure 5.6 Correlations of total arsenic and total selenium in urine samples of different groups: ethnic group (Asian (A), Somali (B) and White (C)), fasting group: RF1 (D) and RF2 (E).* r values for the Somali and the RF2 groups are shown after the removal of outliers.

5.3.3 Arsenic speciation analysis in urine samples

The actual concentrations of arsenic species detected in all different groups were expressed in µg As/g creatinine. The values of arsenic species (AB, DMA) were taken from Chapter 3 and Chapter 4, for ethnic groups and fasting group, respectively.

Arsenic species concentrations also showed a positive correlation with selenium levels in urine samples of the different groups. For AB only two groups (Asian and RF1) showed a positive correlation before (Asian, r = 0.7, *P* = 0.00; RF1, r = 0.4, *P* = 0.02) and after (Asian, r = 0.6, *P* = 0.00; RF1, r = 0.5, *P* = 0.00) creatinine adjustment. This positive correlation was also seen for DMA in all groups before and after creatinine adjustment, with the exception of the Somali group. Before creatinine adjustment, the correlation coefficients and *P*-values are as follows: Asian (r = 0.8, *P* = 0.00), White (r = 0.5, *P* = 0.04), RF1 (r = 0.3, *P* = 0.08) and RF2 (r = 0.5, *P* = 0.01). After creatinine adjustment, the correlation coefficients and *P*-values are as follows: Asian (r = 0.8, *P* = 0.00), White (r = 0.5, *P* = 0.02), RF1 (r = 0.4, *P* = 0.01) and RF2 (r = 0.4, *P* = 0.02). RF1 showed a positive correlation for DMA only after creatinine adjustment. Because MA was not detected in many samples and inorganic arsenic species were not detected in all samples of the different groups, a correlation was not feasible.

5.4 Discussion

As was discussed in Chapters 3 and 4, the majority of the volunteers have similar levels of urinary arsenic within each group, with exception of few volunteers.

This is also applicable to urinary selenium levels of all volunteers in the different groups that are presented in this Chapter (Fig 5.2 and 5.3). The selenium levels that were identified as outliers were all within the normal range [10 – 100 µg/l, Wang et al., (2001); Tiez, (1983)], but only two values (108.5 and 110.2 µg/l, see Table 5.3 and 5.4) were high. However, even these two values are also close to the highest value in the normal range. Importantly, the overall trend of the study is not affected by the few outliers seen, since the exclusion or inclusion of these outliers does not result in any significant changes in the statistical outcome. The individual data of all volunteers in all the different groups for As:Se ratios (Fig 5.4) were found to be clustered around their medians. Only one group (RF2) showed two values as outliers. This suggests the similarity of these ratios among each group, despite the ethnic differences and different condition regarding fasting and non-fasting.

After creatinine adjustment (Fig. 5.3), the outliers either disappeared or were reduced among all the groups. This is in agreement with what reported by Vahter et al., (2006a), that the concentrations of biomarkers in urine are highly dependent on the dilution of the sample. Therefore, the need to adjust this using specific gravity or creatinine is important. The same trend was also established previously for total arsenic in Chapters 3 and 4 for ethnic groups and the fasting group, respectively.

The interesting finding of this study is that a relatively constant As:Se ratio (Table 5.2) was obtained for the single volunteer for a period of one year, and this only alters after recent seafood consumption. Further observation that a similar As:Se ratio is observed for not only the different groups studied here (Table 5.3, 5.4, 5.5, 5.6 and 5.7), but also agrees with values that were calculated from data presented (Table 5.9 and Fig.

5.5) in other studies (UK study with 200 volunteers, German study with 159 volunteers and a Brazilian study with 4 volunteers). Indeed, the As:Se ratio is also relatively constant within a single day as was evident from the analysis of urine samples collected at the beginning of a fast and after an approximately 12 hour fast for 29 volunteers. Although the specific concentration of arsenic and selenium varies significantly between the different groups studied here and previous studies with normal population, the ratio of As:Se determined was found to be very similar with the exception of the Somali group. The average ratio of As: Se (0.7) in Asian and White populations from Leicester (UK) observed in this study and calculated from a previous study of 200 people from the UK (White et al., 1998) suggests that the ratio of these two elements is also similar in the general UK population. In the case of exposed and unexposed population to high arsenic level, the concentration of selenium does not vary greatly between the various groups studied here and by others. However, large variations in arsenic levels can be observed because of exposure to arsenic through consumption of fish or arsenic-contaminated drinking water. Thus, these two factors are the most important factors for variations in the As:Se ratio.

It is tempting to suggest that the As:Se ratio determined in the current study can be considered the baseline level for the As:Se ratio in unexposed populations and can be used as a reliable biomarker of arsenic and selenium level in humans rather than relying solely on arsenic or selenium levels separately. However, the baseline value should be used with caution, because the ratio for one of the groups (Somali) is significantly different from the other groups studied here. Nevertheless, it appears the ratio may provide a more complete picture of arsenic and selenium status in humans

when used in conjunction with the actual concentration of the two elements coupled with background knowledge of the individual's recent food intake.

Dietary intake of arsenic and selenium may play a role in the observed ratio. In the UK, the average total daily dietary intake is 67 µg As/day (MAFF, 2006) and the recommended daily dietary intake of selenium is 60-70 µg Se/day (Rayman, 2002). The dietary intake ratio of As:Se in the UK population is therefore likely to be approximately 1.0. It may be possible that similar intake of these two elements results in a similar pattern of urinary excretion resulting in a ratio of approximately 1.0 for urinary As:Se. The observation that the ratio is also close to one for volunteers from Brazil, suggests that despite geographical and dietary differences the ratio is also similar although the actual levels of arsenic and selenium reported in the study from Brazil is very different from this study. The As:Se of approximately 1.0 was also calculated for a German study with 159 volunteers (Heitland and Koster, 2006). Although diet may play a role, as to if there is a biological reason for the relatively constant As:Se ratio observed in this study is not clear. If a biological process exists in maintaining a ratio of approximately 1.0 between arsenic and selenium, then it suggests that arsenic intake may also be important for human health. This would be in agreement with a previous suggestion which indicated that intake of trace amounts of arsenic may be necessary for metabolism (ATSDR 2000).

The other interesting finding in this study is the positive correlation between total arsenic and selenium in urine samples of all the groups investigated in this thesis. These correlations between total arsenic and selenium in urine samples were established before and after creatinine adjustment. This is the first time that a positive

correlation between total arsenic and selenium has been established among unexposed population.

Two previous studies (Hsueh et al., 2003, Christain et al., 2006) reported a positive correlation between arsenic and selenium in urine samples of exposed populations. However, this correlation was questioned by Vahter et al., (2006a), because the urine samples were not adjusted with creatinine in theses two studies. When analysing urine samples, the variation in the dilution of urine samples can be controlled by creatinine or specific gravity adjustment (Suwazono et al., 2005). Therefore, Vahter et al., (2006a) suggested that the correlation observed by Hsueh et al., (2003) and Christain et al., (2006) before creatinine adjustment, could arise merely from the dilution effect. The samples used in the two studies were neither adjusted with creatinine nor with specific gravity. Vahter et al., (2006b) found a significant correlation between urinary arsenic and selenium before adjustment, in their analysis of urine samples from Bangladeshi population exposed to high levels of arsenic through their drinking water. However, this correlation disappeared after specific gravity adjustment.

Miyazaki et al., (2003) established a negative correlation between arsenic and selenium in urine samples of exposed populations. However, this negative correlation was questioned by Christian et al., (2006) who suggested that this could be because Miyazaki et al., (2003) did not adequately control a range of factors with the exception of gender. However, unlike Hseuh et al., 2003 and Christian et al., (2006) the study by Miyazaki et al., (2003) adjusted the concentrations of arsenic and selenium in their urine samples with creatinine before the correlation was established between arsenic

and selenium. In light of the recent study by Vahter et al., (2006a) the negative correlation observed by Miyazaki et al., (2003) is more likely to be reliable.

However, there is a disagreement between Vahter et. al., (2006b) where no correlation was found after specific gravity adjustment, and Miyazaki et al., (2003) where creatinine adjustment revealed a negative correlation. The reason for this disagreement is not clear and one cannot rule out the possibility that creatinine and specific gravity could have different effect on the correlation.

There is controversy in the literature regarding as to if there is a positive correlation between urinary arsenic and selenium for exposed population. Findings reported here do show a positive correlation between total arsenic and selenium and between selenium and the arsenic species (AB and DMA) among unexposed populations.

5.4 Conclusion

The As:Se ratio data in this Chapter were analysed for each individual within different groups. As:Se ratios for only a few individuals significantly deviated from the rest of the population within each group. However, inclusion or exclusion of these values does not influence the outcome. For the first time a baseline value for urinary As:Se ratio was established for an unexposed population. The intra-individual variation of As:Se ratio remains relatively constant over time, as determined by monitoring a volunteer over a period of one year, and is only affected by recent seafood consumption. Similar ratios were also found after calculation of arsenic and selenium

levels reported in a previous study of 200 people in the UK, and it agrees with values calculated for 159 volunteer from Germany and a small group of volunteers from Brazil. Besides seafood consumption, this study revealed that ethnicity may also result in a significant deviation in this ratio. Seafood consumption and exposure to arsenic through drinking water results in an increase in the ratio to between 2.0 to 9.6. The Somali ethnic group showed a low As:Se ratio (0.4) which can be explained as being due to the lower level of arsenic in their urine. The established baseline value of As:Se ratio in this study among unexposed volunteers from Leicester city may also apply to the general population in UK, assuming that arsenic level is similar in their diet and drinking water. However, a larger study needs to be carried out to confirm that this value can be applied to unexposed populations across UK, Europe and the rest of the world.

This study demonstrated a positive correlation between urinary arsenic and selenium levels amongst unexposed UK population. The fact there is a positive correlation between arsenic and selenium and also that the ratio of these elements remains very similar, with the exception of seafood consumption, between different groups strongly supports the previous studies, which have reported that these two elements interact in the human body such as the involvement of selenium in the methylation of arsenic. When the correlation between arsenic and selenium is not positive and the As:Se ratio alters significantly, despite taking into consideration seafood consumption and other sources of dietary arsenic, it may be a warning sign requiring further investigation.

This study can not provide an explanation as to if there is a biological mechanism that results in the attainment of the As:Se of approximately 1.0 in populations unexposed to arsenic, nor can it determine whether selenium supplement in food or water can be used to restore this ratio in populations exposed to arsenic. However, this finding should be considered as a first step in evaluating the relationship between arsenic and selenium in humans.

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6 Overall conclusion

Suitable methods were developed and/or modified to apply the ICP-MS technique for determination of arsenic levels and speciation of human urine and relate this to different factors such as ethnicity and fasting. The method developed has been successfully applied to establish a baseline arsenic level in human urine samples from volunteers in the United Kingdom who are only exposed to background levels of arsenic. Moreover, a baseline value for As:Se ratio was also determined for the first time. The ICP-MS methods used in this thesis allows establishing a basal data for epidemiological studies.

In addition to ICP-MS, GF-AAS was also used for the analysis of arsenic in human urine, hair and fingernails. Based on the studies reported in this thesis, ICP-MS has proved to be the more powerful technique compared to GF-AAS, regarding total and speciation analysis for arsenic in human urine, especially at low levels.

Arsenic levels in urine, hair and fingernails samples presented in this thesis were analysed for each individual within different groups. Only a few individuals showed major deviation from the rest of the population within each group. However, inclusion or exclusion of these values does not alter the statistical significance of the outcome and the conclusions of the study.

For the first time, a study has been performed with 63 volunteers to explore if there are ethnic differences in arsenic metabolism reflected by differences in levels of arsenic in nail, hair and urine samples. The results obtained did not reveal any statistically significant ethnic differences in the level of arsenic in hair samples. However, there is a significant difference in arsenic concentration in fingernails and in

urine samples of one particular ethnic group (Somali-Black-African) compared to two other groups (Whites and Asian) residing in the same city (Leicester, UK). Furthermore, ethnic difference in urinary arsenic species was found. The study revealed that the Somali ethnic group has a statistically significant difference in the percentage of DMA and AB percentages compared to Whites and Asians. This provides evidence that ethnic difference exist in urinary arsenic excretion patterns. This may be due to differences in genetics, diet or a combination of both dietary and genetic factors.

No major difference in the total level of urinary arsenic in urine samples collected at the beginning and end of 12 hours long fast in a study carried out with 29 volunteers. However, the frequency of detection of MA at the end of fasting period, suggest that the human body, during the daytime fasting period, favours the removal of the most toxic methylated arsenic species detected in this study. It is therefore concluded, that fasting does have an effect on the pattern of urinary arsenic excretion that requires further investigation especially in arsenic exposed regions of the world such as Bangladesh and India.

Relationship between two closely related metalloids, arsenic and selenium, has not been previously investigated with unexposed population, although studies with animals and exposed populations suggest interaction between them. Results presented in this thesis shows that urinary arsenic and selenium are positively correlated amongst a group of unexposed volunteers from Leicester, UK. The urinary As:Se ratio is not influenced by fasting, although ethnicity has a significant effect as one of the groups (Somali) showed a significantly different value for this ratio. The low arsenic level

explains the lower As:Se ratio for the latter group. Other than the Somali group, the vast majority of the population in the UK and in other countries display a constant As:Se ratio that is close to one. This together with the relative constancy in this value observed over a one year period with one volunteer raises the possibility that the As:Se ratio could be considered as a potential biomarker in human urine. The ratio may provide a more complete picture of the urine analysis and the relationship between arsenic and selenium, then just measuring the individual concentrations of these elements. This study cannot explain the underlying reason behind the attainment of the highly constant As:Se ratios (approx. 1.0) in unexposed population, nor can it determine whether selenium supplement in food or water could be used restore this ratio in exposed populations. Nevertheless, the finding of this study should be considered as a first step in evaluating the relationship between arsenic and selenium in humans in order to understand the mechanism behind the established ratio and the relationship between these two elements.

7 Future work

Analysis carried out in this study, principally focused on the analysis of arsenic in urine samples. More analysis need to be carried out with other biological samples including blood. Such samples can be employed for further confirmation of the findings of this thesis. Future studies, such as the ones listed below, needs to be carried out to address some of the unresolved issues that have arisen from this project:

(i) Studies with a larger population size, carefully controlled diet, and genetic analysis need to be carried out in order to identify the precise reason for the observed differences between various ethnic groups in terms of levels and arsenic species detected in urine samples. These studies would aim to ascertain as to which of the following factors is responsible for the difference: dietary, genetic or a combination of both.

(ii) More studies need to be conducted to understand the effect of fasting on urinary arsenic excretion pattern in humans. For example, a study can be carried out with volunteers one month before fasting, during the month long fasting, and one month after fasting to fully understand the effect of Ramadan fasting on arsenic metabolism in exposed and unexposed population. Such a study could reveal if exposed people can perform fasting without suffering adverse health effects from arsenic exposure.

(iii) As:Se ratio may be a potential biomarker in human urine analysis to complement the measurement of arsenic and selenium alone. This may provide a better understanding of the correlation between these two elements. In the future, studies with a larger groups of volunteers, with carefully controlled factors such as ethnicity and diet, needs to be carried out to see if the baseline ratio of As:Se reported in this study is maintained. Importantly, research needs to be carried out to see if a biological process is responsible for the As:Se ratio obtained and the positive correlation between arsenic and selenium. Further work with measurements of other biological tissues such as blood, hair and nail will also help in the understanding of the relationship between arsenic and selenium in humans.

8 Appendices

Appendix (2.1): Questionnaire accompanied with a letter to explain to the volunteers what the project was about and how to deal with the sample in terms of collection and storage.

Appendix (2.2): Representive raw data from blank, standards and CRM NIES No.18. by using GF-AAS.

Appendix (2.3): Representive chromatograms from blank, standards and CRM NIES No.18. by using HPLC-ICP-MS

Appendix (3.1): Detailed information (variables) for urine samples collected from Asian volunteers.

Appendix (3.2): Detailed information (variables) for urine samples collected from Somali volunteers.

Appendix (3.3): Detailed information (variables) for urine samples collected from White volunteers.

Appendix (3.4): Dates of collection and measurements, and locations of urine samples analysis from the Asian volunteers.

Appendix (3.5): Dates of collection and measurements, and locations of urine samples analysis from the Somali volunteers.

Appendix (3.6): Dates of collection and measurements, and locations of urine samples analysis from the White volunteers.

Appendix (3.7): Representive chromatograms for blank, standards, and real urine samples from the Asian, Somali and White volunteers.

Appendix (3.8): Dates of collection and measurements, and locations of hair and fingernail samples analysis from the Asian volunteers.

Appendix (3.9): Dates of collection and measurements, and locations of hair and fingernail samples analysis from the Somali volunteers

Appendix (3.10): Dates of collection and measurements, and locations of hair and fingernail samples analysis from the White volunteers

Appendix (4.1): Detailed information (variables) for urine samples collected from the fasting volunteers.

Appendix (4.2): Dates of collection and measurements, and locations of urine samples analysis from the single fasting volunteer.

Appendix (4.3): Dates of collection and measurements, and locations of urine samples analysis from the fasting volunteers, at two time points RF1 and RF2.

Appendix (4.4): Representive chromatograms for blank, standards, and real urine samples from the Ramadan fasting volunteers.

Appendix (5.1): Dates of collection and measurements, and locations of urine samples analysis from the single volunteer monitored for a period of one year.

Appendix (2.1)

A letter and Questionnaire

E.I.Brima BSc MSc AMRSC
Leicester School of Pharmacy
Faculty of Health and Life SciencesThe
Gateway, Leicester, LE1 9BH

Date:

Re: Study of naturally occurring arsenic containing chemicals in urine.

Dear Sir/Madam,

This study is intended to assess the amount of arsenic found in the human urine of people from as broad a cross-section of society as possible. Non-toxic forms of arsenic are present naturally in an individual's urine. A primary objective of this study is to elucidate the source, i.e. diet or natural origin, of arsenic found in human urine, and determine whether diet or ethnic background is a determining factor in the types of arsenic observed.

The information we need from you via the questionnaire attached to this letter and the analysis result will be treated as confidential.

Please use the following procedure for collection of the urine sample:

1. Collect the first morning urine (mid-stream).
2. About half a bottle urine will be sufficient for the tests.
3. Collect the sample into the polyethylene bottle supplied.
4. Keep the sample cool prior to returning it to Eid Brima and return as soon as after collection as possible.

Thank you for your co-operation.

Eid Brima

Building: Hawthorn
Room: 4.1/1.10
Ext: 8286/8834
Mob: 07919140216
Email: ebrima@dmu.ac.uk
Leicester School of Pharmacy
Faculty of Health and Life Sciences
The Gateway, Leicester,
LE1 9BH

Investigation of arsenic in human urine

Samples will remain anonymous (recognized by a code)

Code: **00002004**

Please tick (✓) as appropriate.

* Please refrain from eating fish and seafood for 3 days prior to sample collection.

Gender	Male	☐	Female	☐	Age ☐
Are you currently menstruating?	Yes	☐	No	☐	
Are you pregnant?	Yes	☐	No	☐	
Are you currently taking any prescription medicines?	Yes	☐	No	☐	
Do you participate in sporting activities?	Yes	☐	No	☐	If yes, How many hours per week? ☐
Are you a vegan?	Yes	☐	No	☐	
Are you a vegetarian?	Yes	☐	No	☐	If yes, do you eat fish? Yes ☐ No ☐
Are you a none meat eater?	Yes	☐	No	☐	
How often do you eat fish?	Once a day	☐	Once a week	☐	Once a month ☐ Never ☐ Have you eaten fish recently? Yes ☐ No ☐ If yes, 1 day ago ☐

					2 days ago ☐ 3 days ago ☐ 1 week ago ☐
How often do you eat seafood?	Once a day	☐	Once a week	☐	Once a month ☐ Never ☐ Have you eaten seafood recently? Yes ☐ No ☐ If yes, 1 day ago ☐ 2 days ago ☐ 3 days ago ☐ 1 week ago ☐
How often do you eat Mushrooms?	Once a day	☐	Once a week	☐	Once a month ☐ Never ☐ Have you eaten Mushrooms recently? Yes ☐ No ☐ If yes, 1 day ago ☐ 2 days ago ☐ 3 days ago ☐ 1 week ago ☐
Are you a smoker?	Yes	☐	No	☐	If yes, how many cigarettes per day? 5-10 ☐ 11-20 ☐ >20 ☐
Do you drink alcohol? * 1 unit = ½ pint beer, 1.5 unit = 1 single spirit, 1 unit = 1 glass wine, 1 unit = 1 glass sherry	Yes	☐	No	☐	If yes, how many units* per week? < 1 ☐ 1-5 ☐ 6-10 ☐ 11-20 ☐

Do you drink tea?	Yes	☐	No	☐	If yes, how many cups per day? ☐ How many cups per week? ☐
Do you drink Coffee?	Yes	☐	No	☐	If yes, how many cups per day? ☐ How many cups per week? ☐
Do you drink soft drinks?	Yes	☐	No	☐	If yes, how many litres per week? ☐
Ethnic origin	Asian or Asian British – Bangladeshi	☐	Asian or Asian British - Indian	☐	Asian or Asian British – Pakistani ☐
	Black or black British - African	☐	Black or black British - Caribbean	☐	Other Black Background ☐
	Mixed - Asian and white	☐	Mixed- Black African and white	☐	Other mixed background ☐ Please Specify,……..
	White - British	☐	Other White background	☐	Other ethnic background ☐ Please Specify,……

Thank you for your valuable help with this study.

Appendix (2.2)

Raw data shows the Repreducibility of GF-AAS by meausuring arsenic standard for ten times

Tube	Sample ID	Conc ppb	%RSD	Mean Abs	BG Abs	Readings	
S:MIX	CAL ZERO	0.00	12.8	0.0093	0.0749	0.0101	0.0084
S:MIX	STANDARD 1	9.99	6.1	0.0677	0.0092	0.0706	0.0648
S:MIX	STANDARD 2	29.99	3.0	0.1979	0.0081	0.1937	0.2021
S:MIX	STANDARD 3	59.97	0.5	0.4038	-0.0076	0.4023	0.4053
1:1	042104CTRLAsV	29.34	0.8	0.1946	-0.0077	0.1934	0.1957
1:2	042105CTRLAsV	32.67	1.1	0.2166	-0.0023	0.2148	0.2183
1:3	042106CTRLAsV	32.06	1.1	0.2125	-0.0091	0.2142	0.2109
1:4	042107CTRLAsV	32.34	1.6	0.2144	-0.0111	0.2168	0.2120
1:5	042108CTRLAsV	30.99	2.4	0.2055	-0.0168	0.2089	0.2021
1:6	042109CTRLAsV	32.18	0.1	0.2133	-0.0191	0.2136	0.2131
1:7	042110CTRLAsV	31.26	2.0	0.2072	-0.0145	0.2101	0.2044
1:8	042111CTRLAsV	31.99	3.0	0.2121	-0.0168	0.2165	0.2077
1:9	042112CTRLAsV	28.60	8.6	0.1897	-0.0158	0.1782	0.2012
1:10	042113CTRLAsV	30.29	0.8	0.2009	-0.0151	0.2020	0.1997

Typical raw data from blank, standards , control and CRM NIES No.18 by using GF-AAS

Tube	Sample ID	Conc ppb	%RSD	Mean Abs	BG Abs	Readings	
S:MIX	CAL ZERO	0.00	1.1	0.0093	0.0488	0.0094	0.0093
S:MIX	STANDARD 2	1.00	25.1	0.0087	0.0078	0.0102	0.0071
S:MIX	STANDARD 3	1.50	10.6	0.0144	0.0102	0.0133	0.0155
S:MIX	STANDARD 4	3.01	3.3	0.0346	-0.0131	0.0338	0.0354
1:8	091604CTRLAsV	2.16	0.0	0.0230	0.0076	0.0230	0.0230
1:14	091604CTRLAsV	2.18	11.9	0.0232	0.0030	0.0212	0.0251
1:23	091604CRM200-1%HNO3	0.85	33.4	0.0086	0.0158	0.0066	0.0107
1:24	091604CRM200-1%HNO3	0.64	7.4	0.0065	0.0120	0.0068	0.0061
1:25	091604CRM200-1%HNO3	0.72	7.3	0.0073	0.0137	0.0069	0.0077
1:26	1%v/vHNO3	0.19	34.0	0.0019	-0.0010	0.0015	0.0024

Appendix (2.3)

Representive chromatograms from blank, standards and CRM NIES No.18.

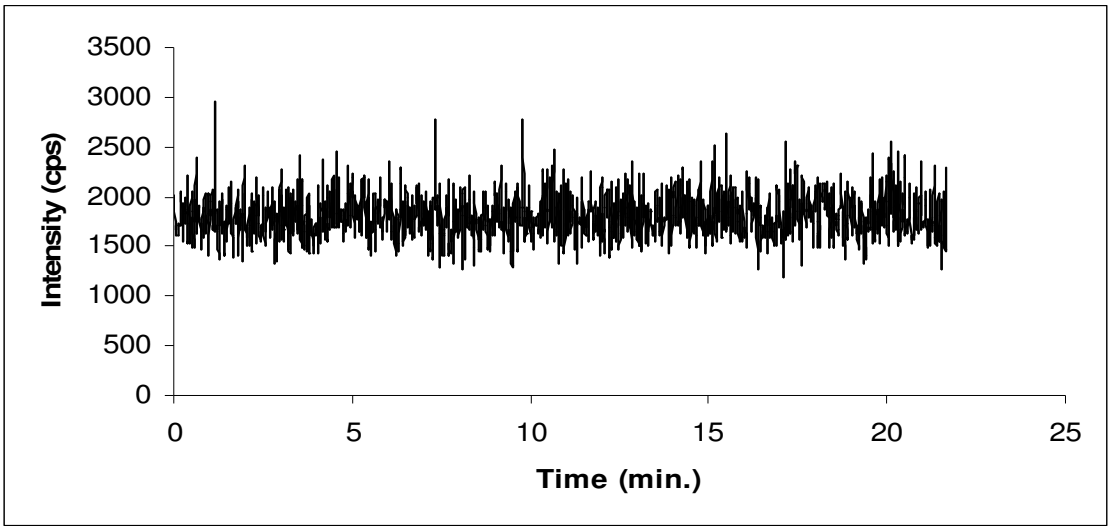

Figure A2.3.1 HPLC-ICP-MS chromatogram of the blank (mobile phase).

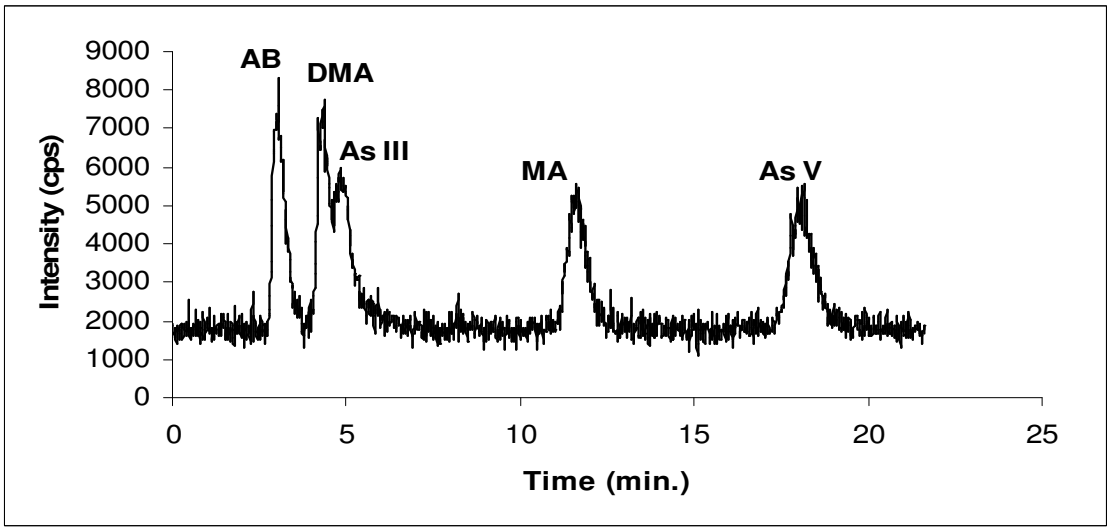

Figure A2.3.2 HPLC-ICP-MS chromatogram of a standard containing 1 µg As/l of each AB, DMA, As (III), MA and As (V).

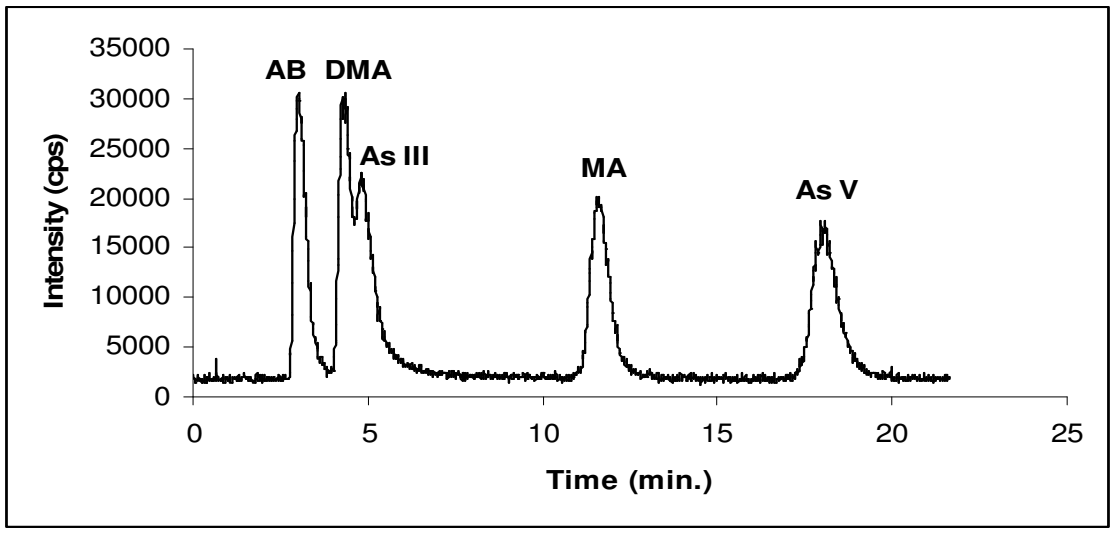

Figure A2.3.3 HPLC-ICP-MS chromatogram of a standard containing 5 µg As/l of each AB, DMA, As (III), MA and As (V).

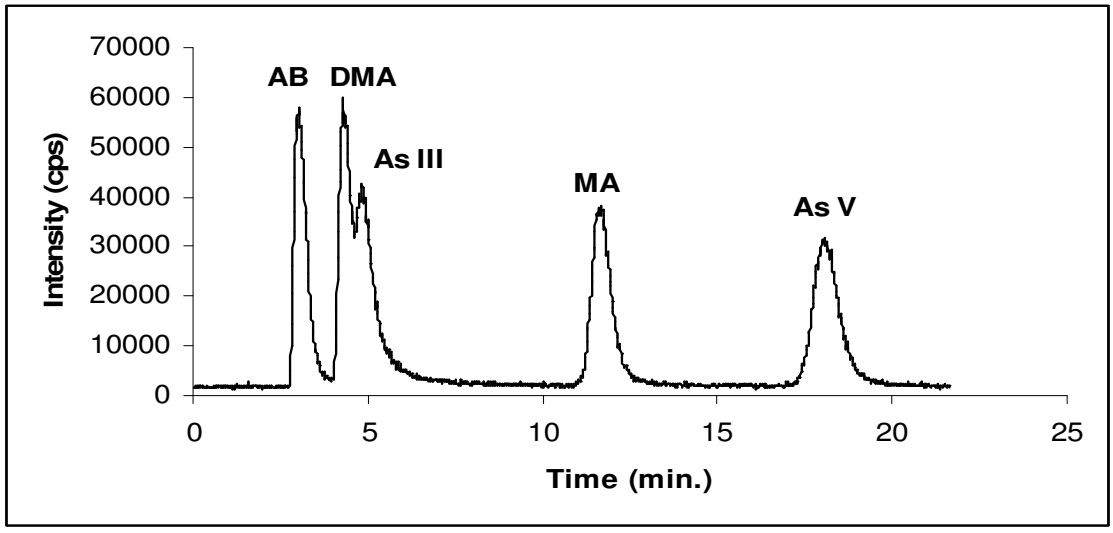

Figure A2.3.4 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l of each AB, DMA, As (III), MA and As (V).

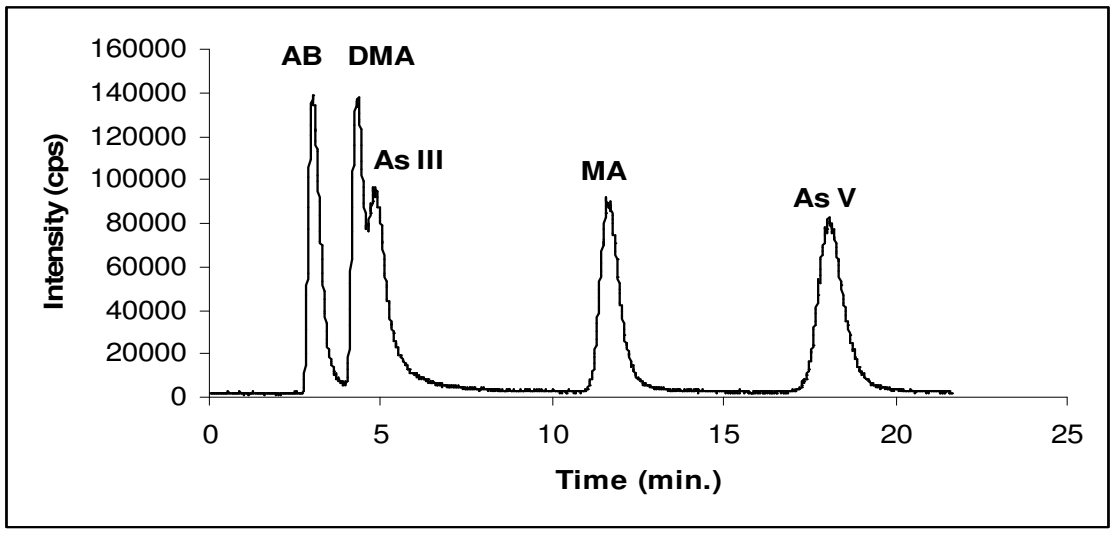

Figure A2.3.5 HPLC-ICP-MS chromatogram of a standard containing 25 µg As/l of each AB, DMA, As (III), MA and As (V).

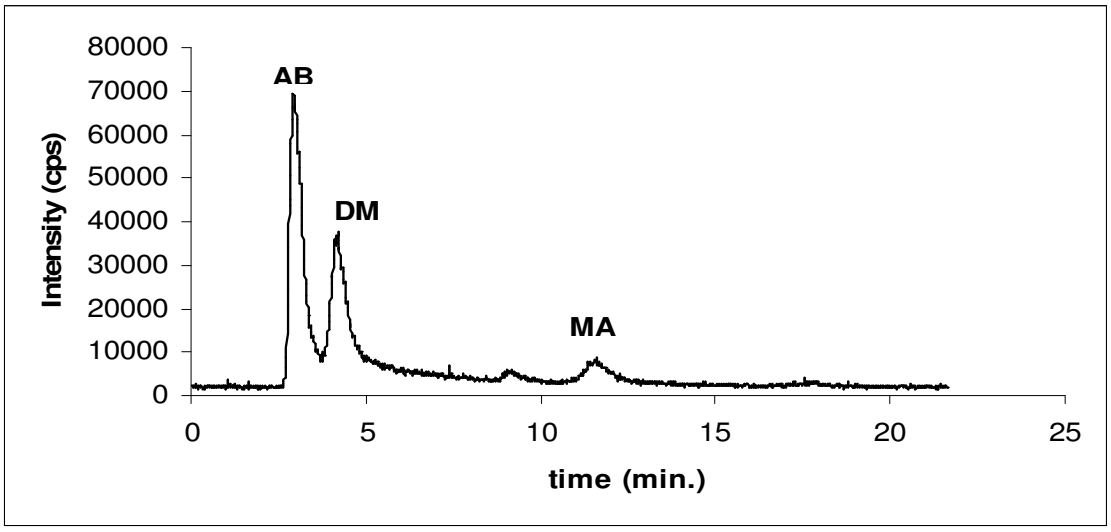

Figure A2.3.6 HPLC-ICP-MS chromatogram of a certified urine sample (CRM NIES No. 18), 5-fold diluted with the mobile phase.

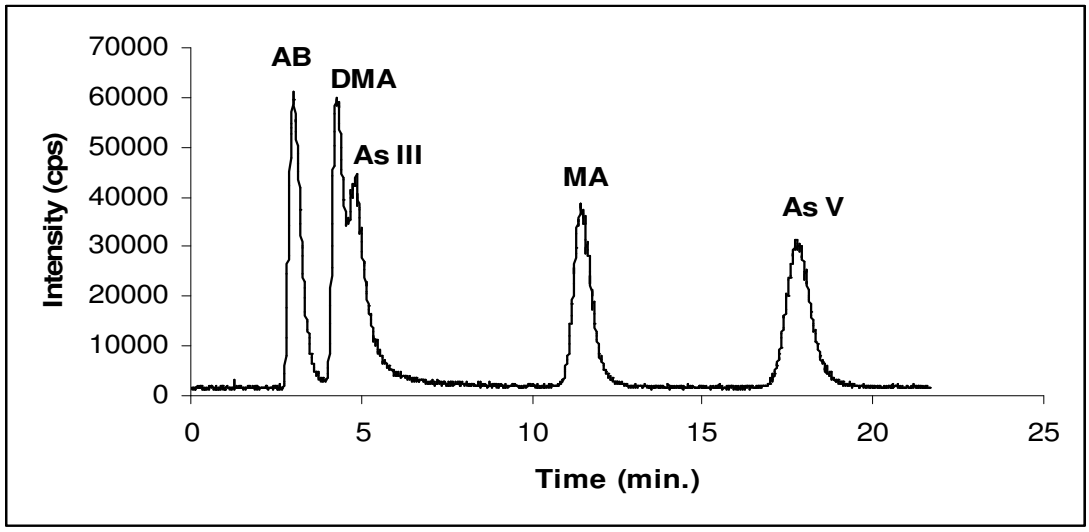

Figure A2.3.4 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppbb1) of each AB, DMA, As (III), MA and As (V).

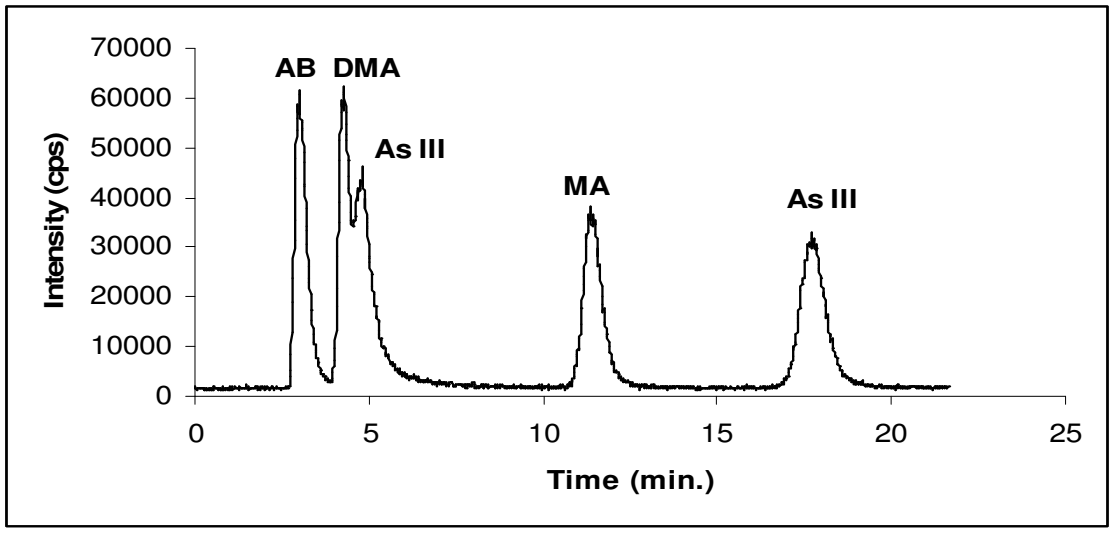

Figure A2.3.4 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppbb2) of each AB, DMA, As (III), MA and As (V). ppb2

Appendix (3.1)

Detailed information (variables) for urine samples (n = 21) collected from Asian volunteers.

Sample No.	Sample Name	Age	Gender	Smoke (cig./wk)	Alcohol (unit/wk)	Soft drink (l/wk)	Tea (cup/wk)	Coffee (cup/wk)	pH
1.	UA1	21	Gender	0	0	1	14	2	5.0
2.	UA2	21	Male	0	0	8	0	4	7.0
3.	UA3	20	Male	0	0	0	0	0	5.0
4.	UA4	20	Male	0	0	0	0	0	7.0
5.	UA5	20	Male	0	0	0	0	0	6.0
6.	UA6	21	Male	0	0	0	0	0	6.0
7.	UA7	18	Male	0	1	0	0	21	5.0
8.	UA8	30	Male	0	0	0	25	3	5.0
9.	UA9	33	Male	0	0	0	25	28	6.5
10	UA10	28	Male	0	0	1	1	0	5.5
11.	UA11	28	Male	0	0	1.5	7	0	5.0
12.	UA12	21	Male	0	0	0	21	0	7.0
13.	UA13	26	Male	10	0	0	21	28	8.0
14.	UA14	26	Male	0	0	1.5	21	0	7.0
15.	UA15	22	Male	0	0	0	14	0	6.0
16.	UA16	-	Male	0	0	0.4	5	0	6.0
17.	UA17	20	Male	0	0	0	20	3	6.5
18.	UA18	33	Male	0	0	0.5	25	0	6.0
19.	UA19	23	Male	0	0	0	12	1	6.5
20.	UA20	26	Female	0	0	2	14	0	6.5
21.	UA21	21	Female	0	0	1	14	4	5.0

Appendix (3.2)

Detailed information (variables) for urine samples (n =22) collected from Somali volunteers.

Sample No.	Sample Name	Age	Gender	Smoke (cig./wk)	Alcohol (unit/wk)	Soft drink (l/wk)	Tea (cup/wk)	Coffee (cup/wk)	pH
1.	US1	35	Male	0	0	3	21	0	5.0
2.	US2	35	Female	0	0	2	3	0	7.0
3.	US3	35	Male	0	0	3	4	0	5.0
4.	US4	24	Male	0	0	0	0	0	7.0
5.	US5	-	Male	0	0	0	21	0	6.0
6.	US6	34	Male	0	0	0	10	6	6.0
7.	US7	-	Male	0	0	0	72	0	5.0
8.	US8	36	Male	0	0	4	14	7	5.0
9.	US9	25	Female	0	0	1	6	1	6.5
10	US10	23	Female	0	0	0	4	0	5.5
11.	US11	27	Male	0	0	0	14	0	5.0
12.	US12	36	Male	0	0	4	14	20	7.0
13.	US13	20	Female	0	0	0	12	3	8.0
14.	US14	36	Female	0	0	0	21	0	7.0
15.	US15	43	Male	0	0	0.5	21	2	6.0
16.	US16	23	Female	0	0	1	14	7	6.0
17.	US17	18	Female	0	0	1	5	3	6.5
18.	US18	49	Male	0	0	0	21	0	6.0
19.	US19	31	Male	0	0	1	21	10	6.5
20.	US20	17	Female	0	0	1	4	3	6.5
21.	US21	19	Female	0	0	4	28	0	5.0
22.	US22	24	Male	0	0	0.5	15	0	5.0

Appendix (3.3)

Detailed information (variables) for urine samples (n = 20) collected from White volunteers.

Sample No.	Sample Name	Age	Gender	Smoke (cig./wk)	Alcohol (unit/wk)	Soft drink (l/wk)	Tea (cup/wk)	Coffee (cup/wk)	pH
1.	UW1	34	Female	10	5	0.5	3	21	5.0
2.	UW2	50	Female	0	1	1	7	28	5.0
3.	UW3	28	Male	0	20	0	10	10	5.0
4.	UW4	30	Male	0	0	0	7	7	5.0
5.	UW5	56	Male	0	5	1	5	30	5.0
6.	UW6	48	Male	0	5	0	7	14	5.0
7.	UW7	40	Female	0	5	0	0	5	6.0
8.	UW8	43	Female	0	10	4	0	50	7.0
9.	UW9	51	Male	0	20	0	0	14	5.0
10.	UW10	37	Female	0	0	0	3	14	5.0
11.	UW11	69	Male	0	20	1	0	35	6.0
12.	UW12	27	Female	20	20	3	7	7	7.0
13.	UW13	27	Female	0	5	0.5	4	8	5.0
14.	UW14	57	Male	0	20	3.5	0	40	5.0
15.	UW15	41	Female	0	10	0.3	21	0	5.0
16.	UW16	22	Female	0	5	1	21	0	5.0
17.	UW17	36	Male	0	5	0.5	25	12	5.0
18.	UW18	57	Male	10	1	2	18	0	5.0
19.	UW19	31	Male	0	20	2	0	15	5.0
20.	UW20	46	Female	0	5	0	35	7	6.0

Appendix (3.4)

Dates of collection and measurements, and locations of urine samples analysis from the Asian volunteers.

Sample name	Date of collection	Date of measurement (**speciation**)- ***at Manchester University***	Date of measurement (**total**)- ***at Hull University***
UA1	01/10/2004	25/02/2005	16/08/2005
UA2	01/10/2004	25/02/2005	16/08/2005
UA3	20/10/2004	25/02/2005	16/08/2005
UA4	20/10/2004	25/02/2005	16/08/2005
UA5	20/10/2004	25/02/2005	17/08/2005
UA6	20/10/2004	25/02/2005	16/08/2005
UA7	25/10/2004	25/02/2005	16/08/2005
UA8	01/11/2004	25/02/2005	16/08/2005
UA9	01/11/2004	25/02/2005	16/08/2005
UA10	03/11/2004	25/02/2005	16/08/2005
UA11	04/11/2004	25/02/2005	16/08/2005
UA12	05/11/2004	25/02/2005	16/08/2005
UA13	09/11/2004	25/02/2005	16/08/2005
UA14	23/11/2004	04/04/2005	16/08/2005
UA15	24/11/2004	04/04/2005	16/08/2005
UA16	30/11/2004	04/04/2005	16/08/2005
UA17	30/11/2004	04/04/2005	16/08/2005
UA18	30/11/2004	04/04/2005	17/08/2005
UA19	01/12/2005	04/04/2005	16/08/2005
UA20	09/12/2004	04/04/2005	16/08/2005
UA21	13/12/2004	04/04/2005	16/08/2005

Appendix (3.5)

Dates of collection and measurements, and locations of urine samples analysis from the Somali volunteers.

Sample name	Date of collection	Date of measurement (**speciation**)- ***at Manchester University***	Date of measurement (**total**)- ***at Hull University***
US1	19/10/2004	04/04/2005	16/08/2005
US2	19/10/2004	05/04/2005	16/08/2005
US3	19/10/2004	05/04/2005	16/08/2005
US4	19/10/2004	05/04/2005	16/08/2005
US5	19/10/2004	05/04/2005	16/08/2005
US6	19/10/2004	05/04/2005	16/08/2005
US7	20/10/2004	05/04/2005	16/08/2005
US8	21/10/2004	05/04/2005	16/08/2005
US9	22/10/2004	05/04/2005	16/08/2005
US10	22/10/2004	05/04/2005	16/08/2005
US11	22/10/2004	05/04/2005	16/08/2005
US12	26/10/2004	05/04/2005	16/08/2005
US13	26/10/2004	05/04/2005	16/08/2005
US14	27/10/2004	06/04/2005	16/08/2005
US15	27/10/2004	06/04/2005	16/08/2005
US16	28/10/2004	06/04/2005	16/08/2005
US17	02/11/2004	06/04/2005	16/08/2005
US18	02/11/2004	06/04/2005	16/08/2005
US19	02/11/2004	06/04/2005	16/08/2005
US20	02/11/2004	06/04/2005	16/08/2005
US21	02/11/2004	06/04/2005	16/08/2005
US22	13/12/2004	06/04/2005	16/08/2005

Appendix (3.6)

Dates of collection and measurements, and locations of urine samples analysis from the White volunteers.

Sample name	Date of collection	Date of measurement (**speciation**)- ***at Manchester University***	Date of measurement (**total**)- ***at Hull University***
UW1	11/10/2004	23/02/2005	16/08/2005
UW2	14/10/2004	23/02/2005	16/08/2005
UW3	14/10/2004	24/02/2005	16/08/2005
UW4	15/10/2004	24/02/2005	17/08/2005
UW5	15/10/2004	24/02/2005	16/08/2005
UW6	18/10/2004	24/02/2005	16/08/2005
UW7	18/10/2004	24/02/2005	16/08/2005
UW8	19/10/2004	24/02/2005	16/08/2005
UW9	22/10/2004	24/02/2005	16/08/2005
UW10	25/10/2004	24/02/2005	16/08/2005
UW11	22/11/2004	24/02/2005	16/08/2005
UW12	25/11/2004	24/02/2005	16/08/2005
UW13	06/12/2004	24/02/2005	16/08/2005
UW14	10/12/2004	11/02/2005	16/08/2005
UW15	13/11/2004	11/02/2005	16/08/2005
UW16	14/11/2004	11/02/2005	16/08/2005
UW17	09/01/2004	11/02/2005	16/08/2005
UW18	12/01/2004	23/02/2005	16/08/2005
UW19	13/01/2005	11/02/2005	16/08/2005
UW20	21/02/2004	23/02/2005	16/08/2005

Appendix (3.7)

Representive figures from blank, standards, CRM NIES No.18, and real urine samples from the Asian, Somali and White volunteers.

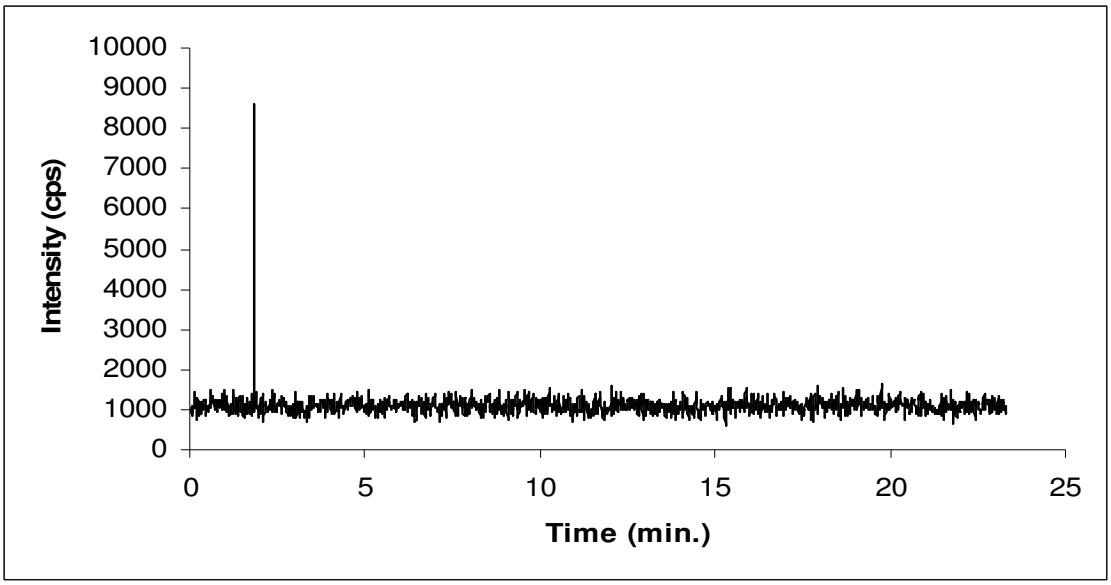

Figure A3.7.1 HPLC-ICP-MS chromatogram of the blank (mobile phase).

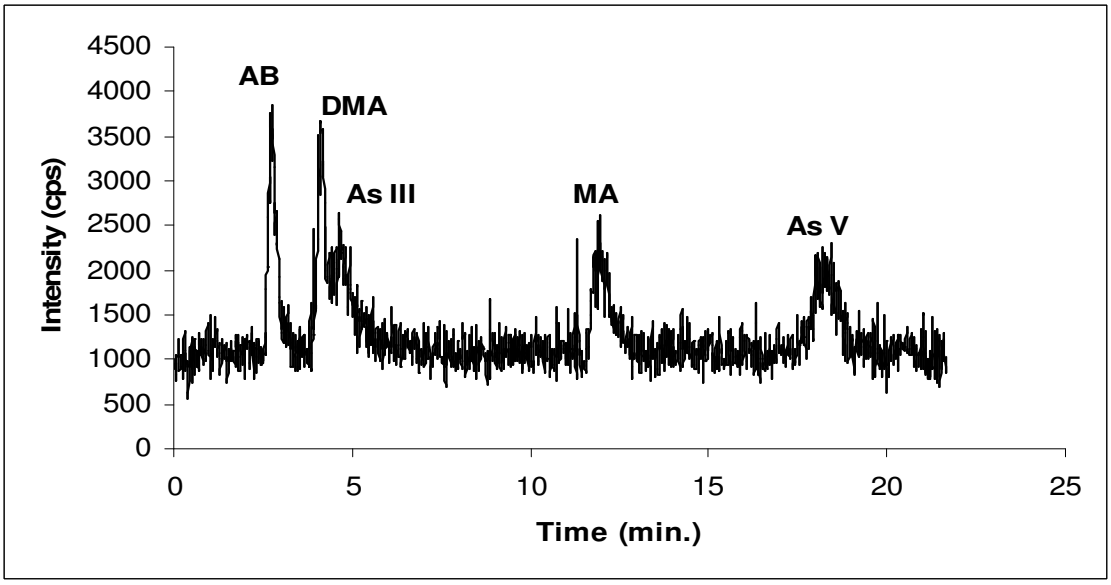

Figure A3.7.2 HPLC-ICP-MS chromatogram of a standard containing 1 µg As/l of each AB, DMA, As (III), MA and As (V).

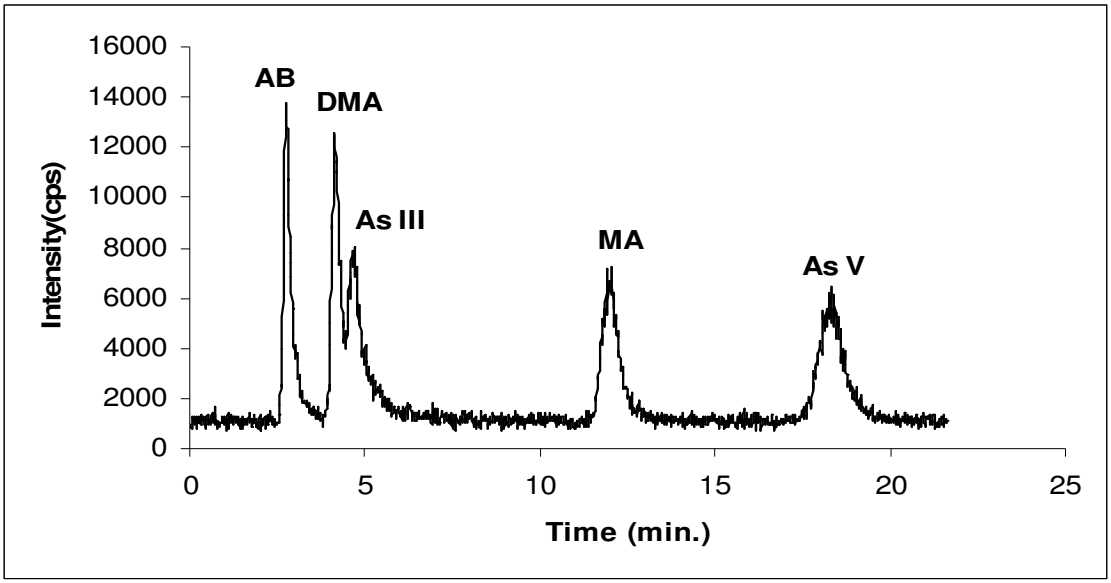

Figure A3.7.3 HPLC-ICP-MS chromatogram of a standard containing 5 µg As/l of each AB, DMA, As (III), MA and As (V).

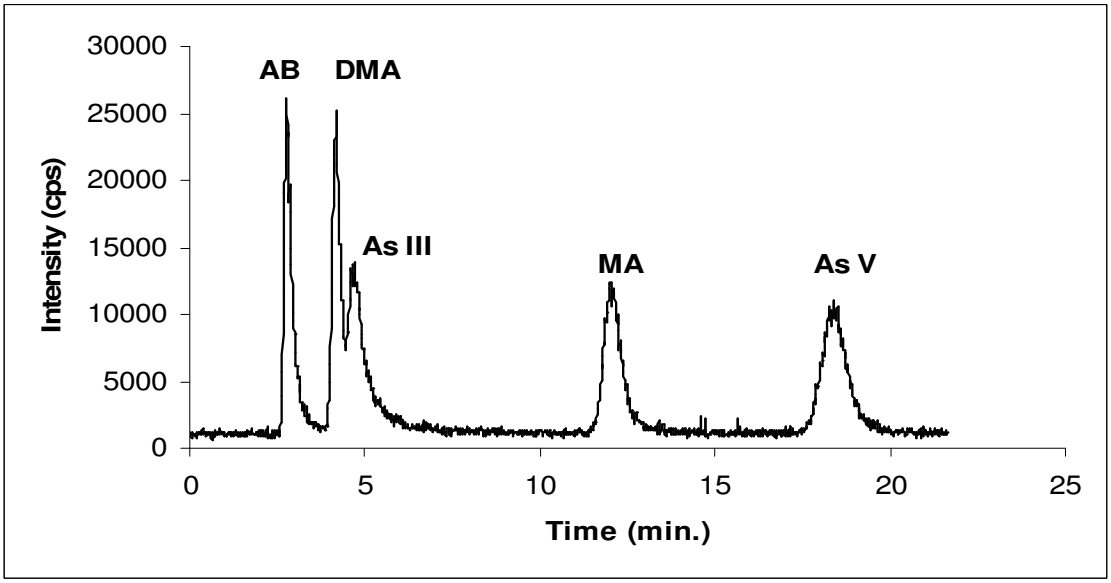

Figure A3.7.4 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l of each AB, DMA, As (III), MA and As (V).

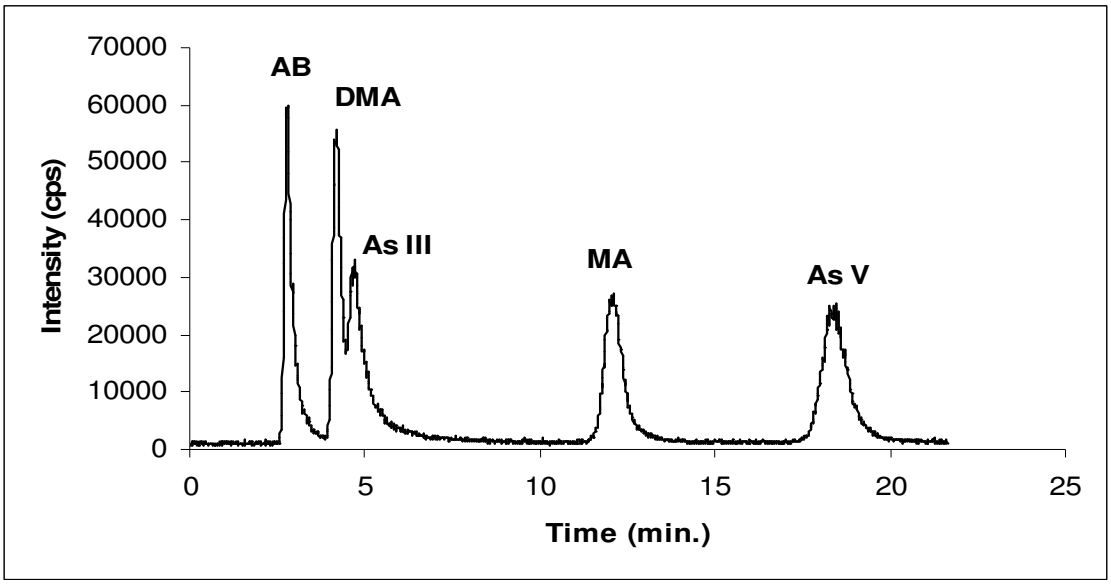

Figure A3.7.5 HPLC-ICP-MS chromatogram of a standard containing 25µg As/l of each AB, DMA, As (III), MA and As (V).

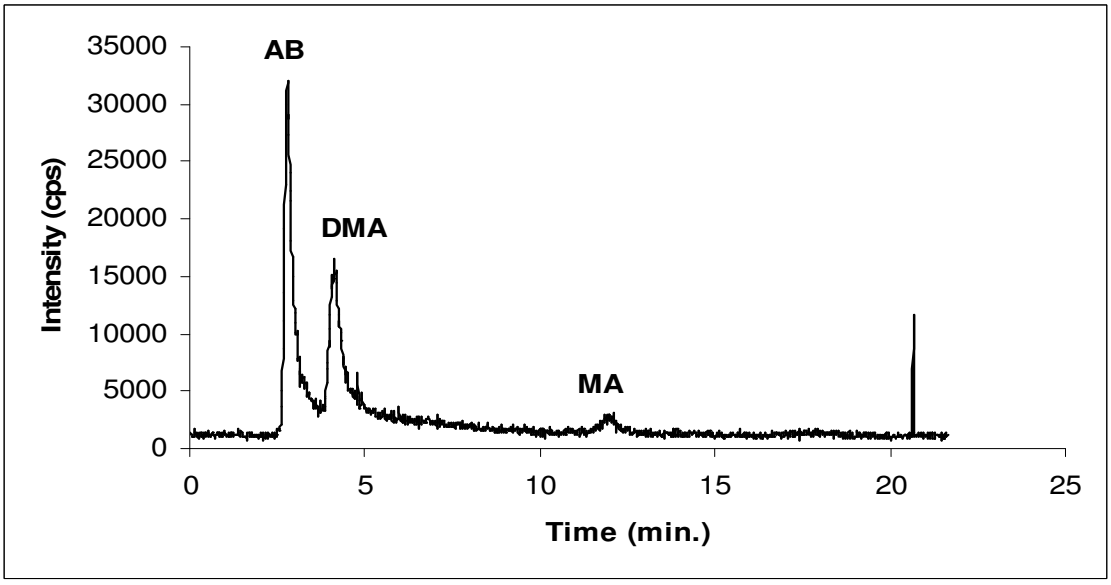

Figure A3.7.6 HPLC-ICP-MS chromatogram of a certified urine sample (CRM NIES No. 18), 5-fold diluted with the mobile phase.

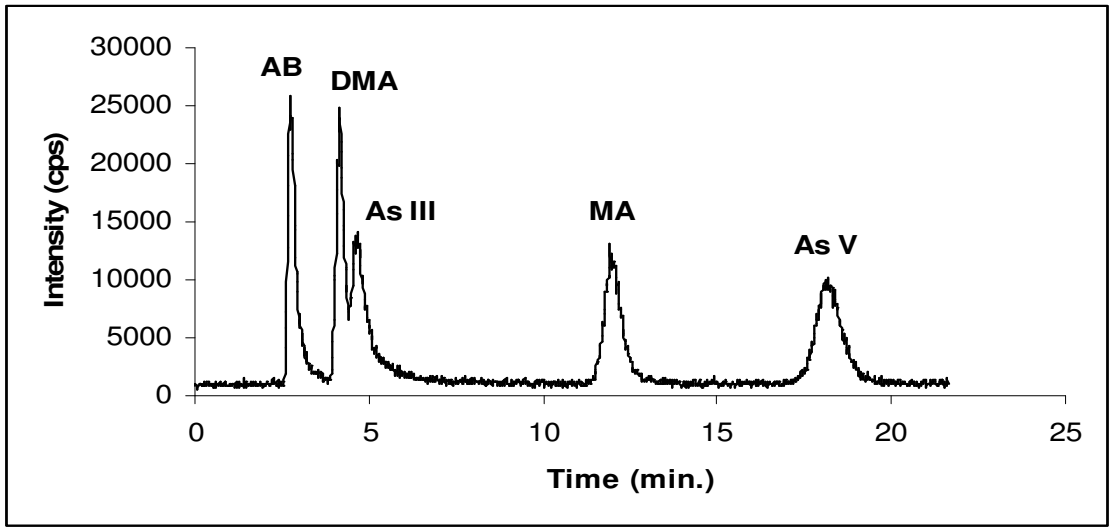

Figure A3.7.7 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppb1) of each AB, DMA, As (III), MA and As (V).

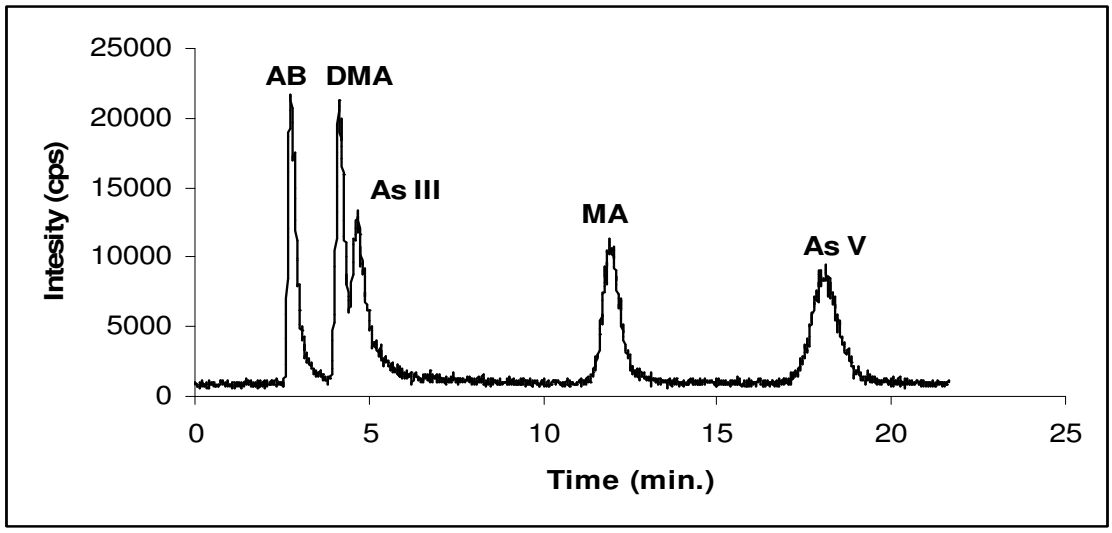

Figure A3.7.8 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppb2) of each AB, DMA, As (III), MA and As (V).

Representive figures of real urine samples from two Asian volunteers

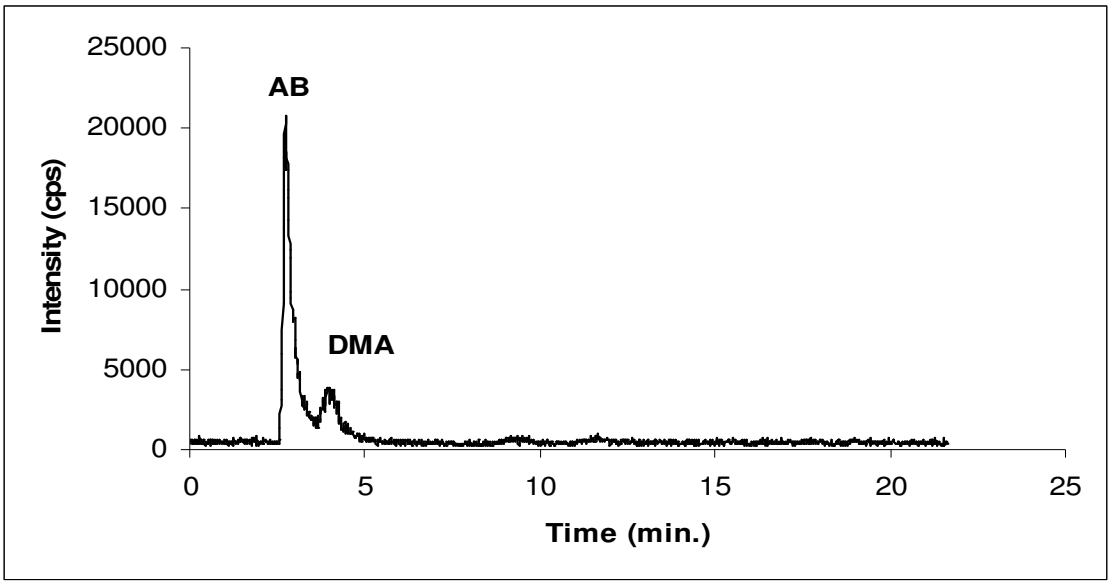

Figure A3.7.9 HPLC-ICP-MS chromatogram of a urine sample (UA5) 5-fold diluted with the mobile phase.

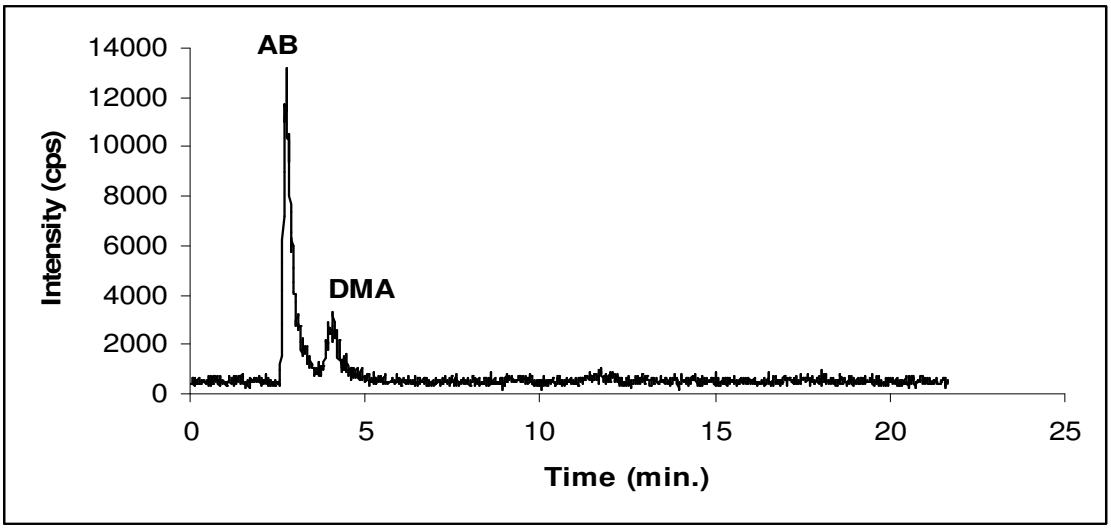

Figure A3.7.10 HPLC-ICP-MS chromatogram of a urine sample (UA3) 5-fold diluted with the mobile phase.

Representive figures of real urine samples from two Somali volunteers

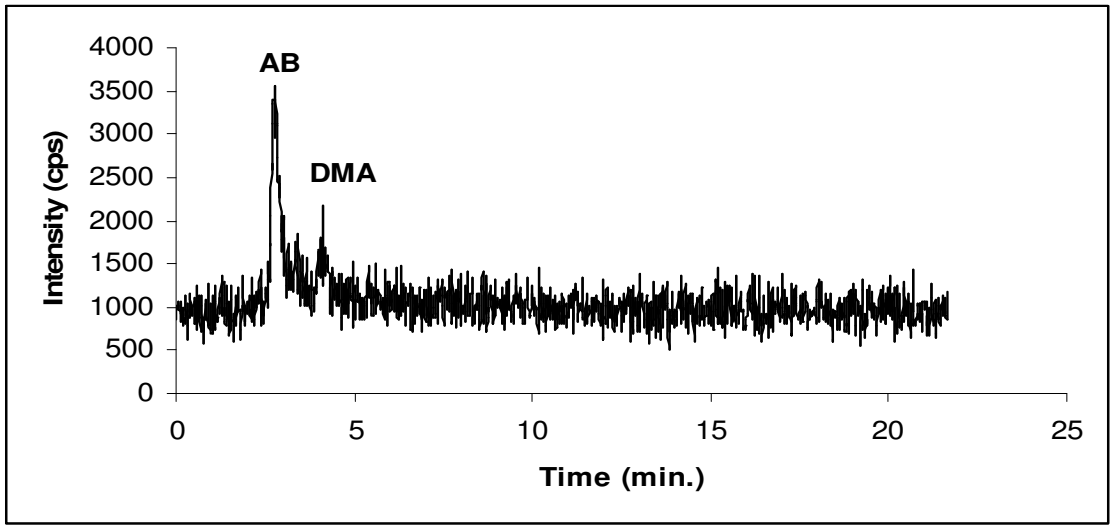

Figure A3.7.11 HPLC-ICP-MS chromatogram of a urine sample (US9) 5-fold diluted with the mobile phase.

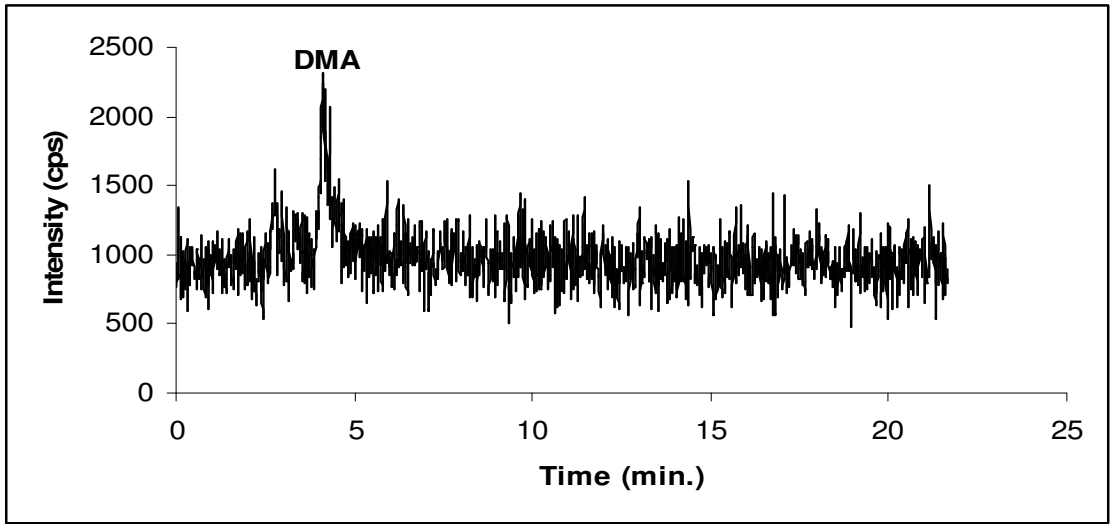

Figure A3.7.12 HPLC-ICP-MS chromatogram of a urine sample (US11) 5-fold diluted with the mobile phase.

Representive figures of real urine samples from two White volunteers

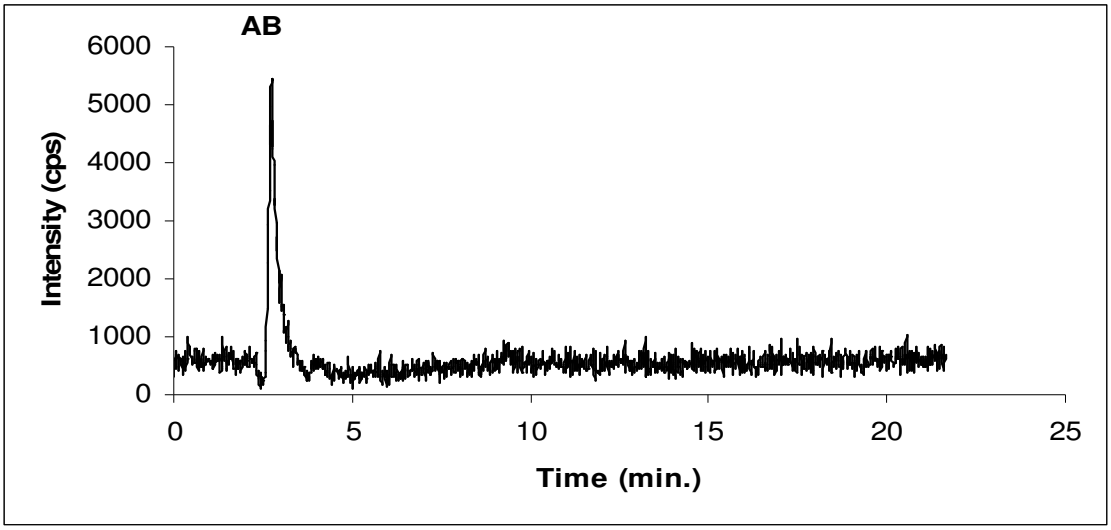

Figure A3.7.13 HPLC-ICP-MS chromatogram of a urine sample (UW13) 5-fold diluted with the mobile phase.

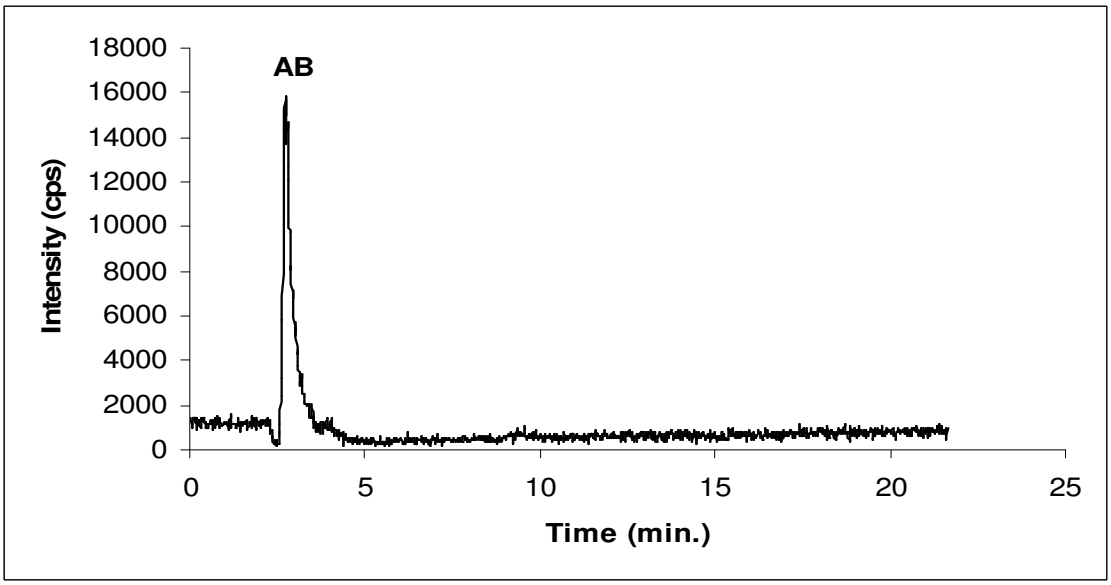

Figure A3.7.14 HPLC-ICP-MS chromatogram of a urine sample (UW4) 5-fold diluted with the mobile phase.

Appendix (3.8)

Dates of collection and measurements, and locations of hair and fingernail samples analysis from the Asian volunteers.

Sample name **Hair**	Sample name **Nail**	Date of collection	Date of digestion	Date of measurement (**total arsenic**)- ***at De Montfort University***
HA1(26)*	NA1(26)*	06/06/2005	03/09/2005	24/10/2005 11/11/2005 (HA1)
HA2(27)	NA2(27)	06/06/2005	03/09/2005	24/10/2005
HA3(28)	NA3(28)	20/05/2005	03/09/2005	24/10/2005 11/11/2005 (HA3)
HA4(29)	NA4(29)	20/05/2005	03/09/2005	24/10/2005 11/11/2005 (NA4)
HA5(32)	NA5(32)	05/08/2005	03/09/2005	24/10/2005
HA6(33)	NA6(33)	09/08/2005	03/09/2005	24/10/2005 11/11/2005 (NA6)
HA7(34)	NA7(34)	09/08/2005	03/09/2005	24/10/2005
HA8(35)	NA8(35)	10/08/2005	03/09/2005	24/10/2005 11/11/2005 (HA8)
HA9(36)	NA9(36)	10/08/2005	03/09/2005	24/10/2005 11/11/2005 (NA9)
HA10(37)	NA10(37)	10/08/2005	03/09/2005	24/10/2005 11/11/2005 (HA10)

* Numbers between brackets are additional names given to hair and fingernail samples, and apply to the following Appendices 3.9 to 3.10. The additional names are read as H26 and N26.

Appendix (3.9)

Dates of collection and measurements, and locations of hair and fingernail samples analysis from the Somali volunteers.

Sample name **Hair**	Sample name **Nail**	Date of collection	Date of digestion	Date of measurement (**total arsenic**)- ***at De Montfort University***
HS1(1)	NS1(1)	13/05/2005	03/08/2005	24/10/2005
HS2(2)	NS2(2)	14/05/2005	03/08/2005	24/10/2005 11/11/2005 (HS2)
HS3(3)	NS3(3)	14/05/2005	03/08/2005	11/11/2005
HS4(4)	NS4(4)	15/05/2005	03/08/2005	24/10/2005 11/11/2005 (HS4)
HS5(5)	NS5(5)	15/05/2005	05/08/2005	24/10/2005
HS6(6)	NS6(6)	16/05/2005	05/08/2005	24/10/2005
HS7(7)	NS7(7)	20/05/2005	05/08/2005	24/10/2005
HS8(8)	NS8(8)	20/05/2005	05/08/2005	24/10/2005 11/11/2005 (NS8)
HS9(9)	NS9(9)	20/05/2005	05/08/2005	24/10/2005 11/11/2005 (HS9)
HS10(10)	NS10(10)	24/05/2005	05/08/2005	24/10/2005
HS11(11)	NS11(11)	24/05/2005	05/08/2005	24/10/2005
HS12(12)	NS12(12)	24/05/2005	07/08/2005	11/11/2005
HS13(13)	NS13(13)	24/05/2005	07/08/2005	24/10/2005
HS14(14)	NS14(14)	24/05/2005	07/08/2005	24/10/2005 11/11/2005 (HS14)
HS15(15)	NS15(15)	30/05/2005	07/08/2005	24/10/2005 11/11/2005 (NS15)

Appendix (3.10)

Dates of collection and measurements, and locations of hair and fingernail samples analysis from the Asian volunteers.

Sample name **Hair**	Sample name **Nail**	Date of collection	Date of digestion	Date of measurement (**total arsenic**)- ***at De Montfort University***
HW1(30)	NW1(30)	03/06/2005	05/09/2005	11/11/2005
HW2(31)	NW2(31)	06/08/2005	05/09/2005	24/10/2005
HW3(38)	NW3(38)	09/08/2005	07/09/2005	11/11/2005
HW4(39)	NW4(39)	09/08/2005	07/09/2005	11/11/2005
HW5(40)	NW5(40)	09/08/2005	07/09/2005	11/11/2005
HW6(41)	NW6(41)	09/08/2005	07/09/2005	11/11/2005
HW7(42)	NW7(42)	09/08/2005	07/09/2005	11/11/2005
HW8(43)	NW8(43)	10/08/2005	07/09/2005	11/11/2005
HW9(44)	NW9(44)	01/09/2005	07/09/2005	11/11/2005
HW10(45)	NW10(45)	02/09/2005	09/09/2005	11/11/2005
HW11(46)	NW11(46)	06/09/2005	09/09/2005	11/11/2005

Appendix (4.1)

Detailed information (variables) for urine samples (n = 58) collected from the fasting volunteers (n = 29) at two time points (RF1* and RF2*).

Sample No.	Sample name (**RF1**)	Sample name (**RF2**)	***Ethnicity****	***Age***	Gender	***Smoke (cig./wk)***	***Alcohol (unit/wk)***	***Soft drink (l/wk)***	***Tea (cup/wk)***	***Coffee (cup/wk***	pH	
											RF1	**RF2**
1.	UNF1	UF1	B	39	Male	0	0	1	10	0	5	5
2.	UNF2	UF2	A	19	Male	0	0	0.5	9	0	5	5
3.	UNF3	UF3	B	37	Male	0	0	1	14	0	5	5
4.	UNF4	UF4	ME	-	Male	0	0	0	28	3	6	5
5.	UNF5	UF5	B	32	Male	0	0	1	14	0	7	6
6.	UNF6	UF6	B	34	Male	0	0	0	4	0	7.5	7
7.	UNF7	UF7	B	32	Male	0	0	2	15	0	6	7.5
8.	UNF8	UF8	B	27	Male	0	0	1	7	0	7	5
9.	UNF9	UF9	ME	39	Male	0	0	6	15	1	6	7
10.	UNF10	UF10	A	42	Male	0	0	2	14	1	7.5	7
11.	UNF12	UF12	ME	26	Male	0	0	1	10	0	6.0	7.5
12	UNF13	UF13	A	33	Male	0	0	2	25	3	6	6
13	UNF14	UF14	A	28	Male	0	0	0	25	28	6	7.5
14	UNF15	UF15	ME	26	Male	0	0	3	15	0	6.5	6
15	UNF16	UF16	NA	42	Male	0	0	0	10	0	6.5	7

Appendix (4.1) coninued

Sample No.	Sample name **(RF1)**	Sample name **(RF2)**	***Ethnicity****	***Age***	Gender	***Smoke (cig./wk)***	***Alcohol (unit/wk)***	***Soft drink (l/wk)***	***Tea (cup/wk)***	***Coffee (cup/wk***	pH	
											RF1	**RF2**
16	UNF17	UF17	NA	39	Male	0	0	0	10	14	6.5	6
17	UNF19	UF19	B	49	Male	0	0	0	21	0	6	6
18	UNF21	UF21	B	37	Male	0	0	2	14	14	6.5	6.5
19	UNF22	UF22	ME	30	Female	0	0	1	7	0	6.5	6.5
20	UNF24	UF24	A	31	Female	0	0	0	0	0	6.5	6
21	UNF25	UF25	A	28	Male	0	0	1	1	0	6	6
22	UNF26	UF26	ME	26	Male	30	0	2	21	1	7.5	7.5
23	UNF27	UF27	B	22	Male	0	0	2	14	0	7	6
24	UNF28	UF28	B	35	Male	0	0	2	2	0	6.5	7
25	UNF29	UF29	A	21	Male	0	0	1.5	7	0	6	6.5
26	UNF30	UF30	NA	31	Female	0	0	3	4	3	6	6.5
27	UNF32	UF32	A	26	Male	0	0	0	3	0	7	6.5
28	UNF33	UF33	ME	37	Male	0	0	1	7	0	7	6
29	UNF34	UF34	A	26	Male	10	0	0	21	28	8	7

*Each pair samples were collected from the same volunteer, at the beginning of fasting period **RF1** (UNF) and at the end of fasting period **RF2** (UF).

*A = Asian or Asian British –(Indian,Bangladesh or Pakistani); B = Black or Black British – African; NA = North African;
ME = Middle East

Appendix (4.2)

Dates of collection and measurements, and locations of urine samples analysis from the single fasting volunteer.

Sample No.	Sample name **RF1***	Sample name **RF2***	Date of collection	Date of measurement (**speciation of arsenic**)- ***at Manchester University***	Date of measurement (**total arsenic**)- ***at Hull University***
1.	UNC1F1	UC1F1	05/11/2004	30/06/2005	16/08/2005
2.	UNC1F2	UC1F2	06/11/2004	30/06/2005	16/08/2005
3.	UNC1F3	UC1F3	07/11/2004	30/06/2005	16/08/2005
4.	UNC1F4	UC1F4	08/11/2004	30/06/2005	16/08/2005
5.	UNC1F5	UC1F5	09/11/2004	04/07/2005	16/08/2005

***** Each pair samples were collectedon the same day , at the beginning of fasting period **RF1** (UNF) and at the end of fasting period **RF2** (UF).

Appendix (4.3)

Dates of collection and measurements, and locations of urine samples analysis from the fasting volunteers, at two time points RF1 And RF2.

Sample name **(RF1)**	Sample name **(RF2)**	Date of collection #	Date of measurement (**speciation**)- ***at Manchester University***	Date of measurement (**total**)- ***at Hull University***
UNF1	UF1	24/10/2004	25/05/2005	16/08/2005
UNF2	UF2	27/10/2004	25/05/2005	16/08/2005
UNF3	UF3	27/10/2004	25/05/2005	16/08/2005
UNF4	UF4	27/10/2004	26/05/2005	16/08/2005
UNF5	UF5	27/10/2004	26/05/2005	16/08/2005
UNF6	UF6	28/10/2004	26/05/2005	16/08/2005
UNF7	UF7	28/10/2004	26/05/2005	16/08/2005
UNF8	UF8	29/10/2004	13/06/2005	16/08/2005
UNF9	UF9	29/10/2004	26/05/2005	16/08/2005
UNF10	UF10	29/10/2004	27/05/2005	16/08/2005
UNF12	UF12	01/11/2005	27/05/2005	16/08/2005
UNF13	UF13	01/11/2005	13/06/2005	16/08/2005
UNF14	UF14	01/11/2005	14/06/2005	16/08/2005
UNF15	UF15	01/11/2005	14/06/2005	16/08/2005
UNF16	UF16	01/11/2005	14/06/2005	16/08/2005
UNF17	UF17	01/11/2005	14/06/2005	16/08/2005
UNF19	UF19	03/11/2005	14/06/2005	16/08/2005
UNF21	UF21	03/11/2005	15/06/2005	16/08/2005
UNF22	UF22	03/11/2005	15/06/2005	16/08/2005
UNF24	UF24	03/11/2005	15/06/2005	16/08/2005
UNF25	UF25	03/11/2005	15/06/2005	16/08/2005
UNF26	UF26	03/11/2005	28/06/2005	16/08/2005
UNF27	UF27	04/11/2005	28/06/2005	16/08/2005
UNF28	UF28	04/11/2005	29/06/2005	16/08/2005
UNF29	UF29	04/11/2005	29/06/2005	16/08/2005
UNF30	UF30	05/11/2005	29/06/2005	16/08/2005
UNF32	UF32	05/11/2005	29/06/2005	16/08/2005
UNF33	UF33	04/11/2005	29/06/2005	16/08/2005
UNF34	UF34	09/11/2005	29/06/2005	16/08/2005

Appendix (4.4)

Representive chromatograms from blank, standards, CRM NIES No. 18 and real urine samples from Ramadan fasting group.

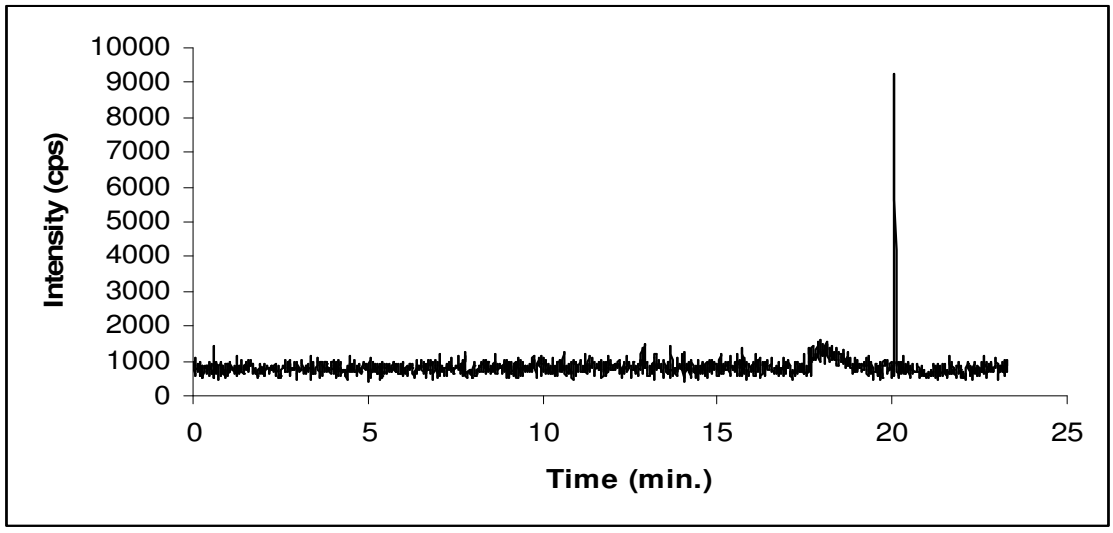

Figure A4.4.1 HPLC-ICP-MS chromatogram of the blank (mobile phase).

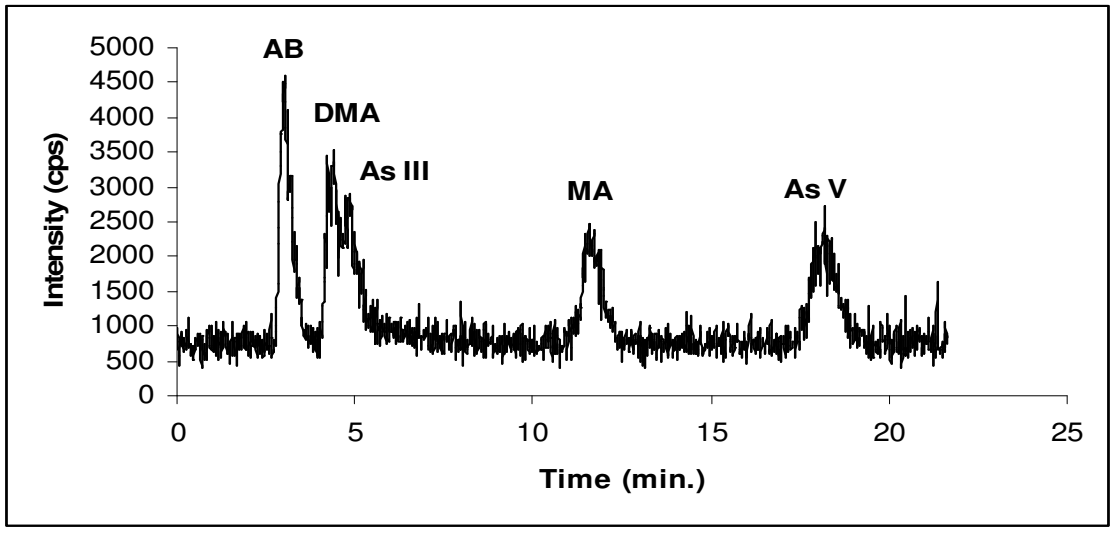

Figure A4.4.2 HPLC-ICP-MS chromatogram of a standard containing 1 µg As/l of each AB, DMA, As (III), MA and As (V).

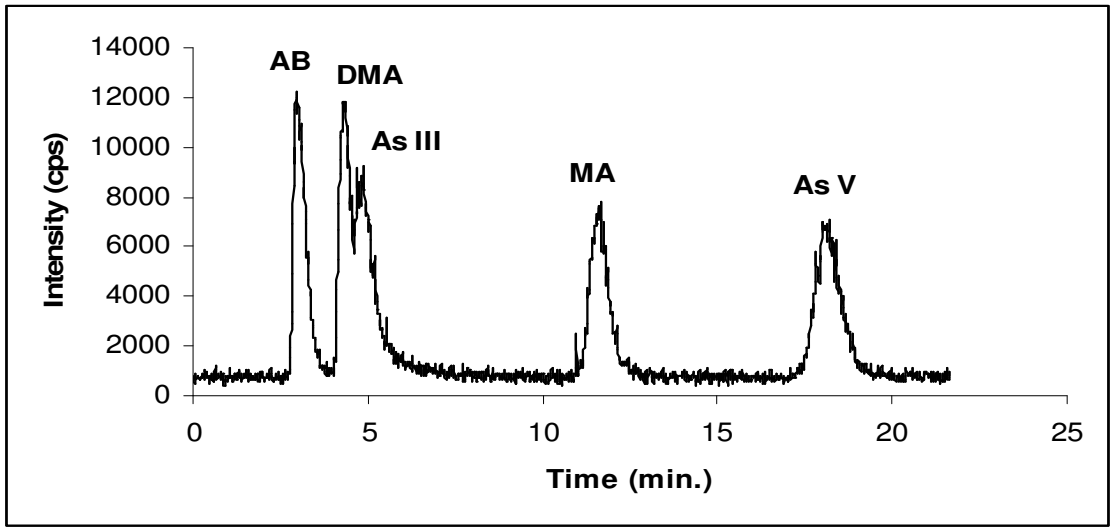

Figure A4.4.3 HPLC-ICP-MS chromatogram of a standard containing 5 µg As/l of each AB, DMA, As (III), MA and As (V).

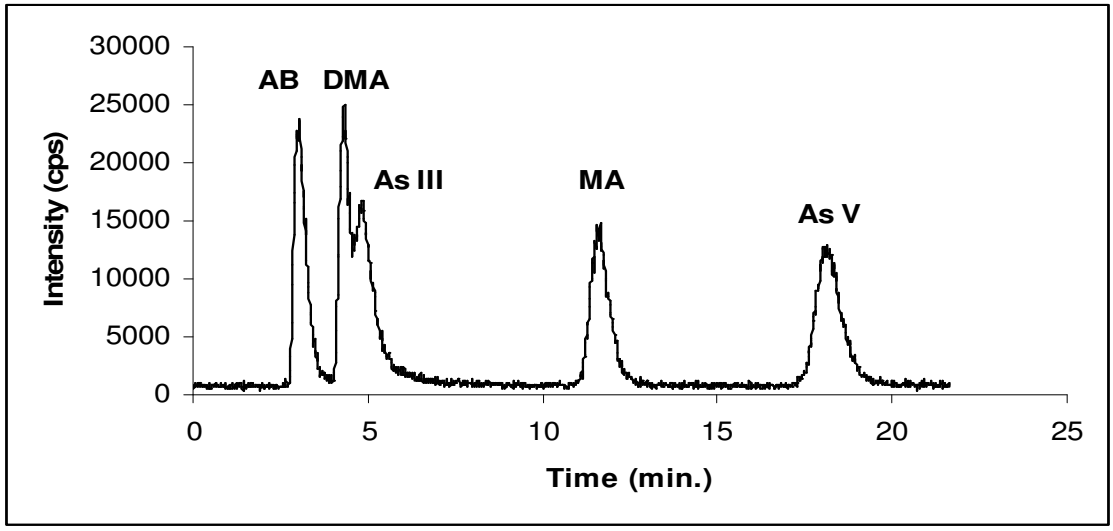

Figure A4.4.4 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l of each AB, DMA, As (III), MA and As (V).

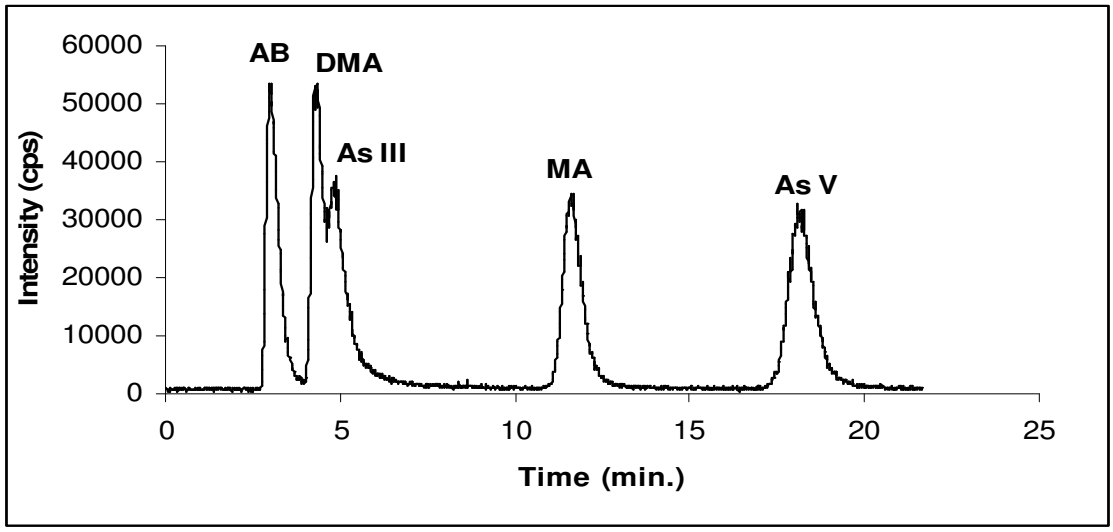

Figure A4.4.5 HPLC-ICP-MS chromatogram of a standard containing 25 µg As/l of each AB, DMA, As (III), MA and As (V).

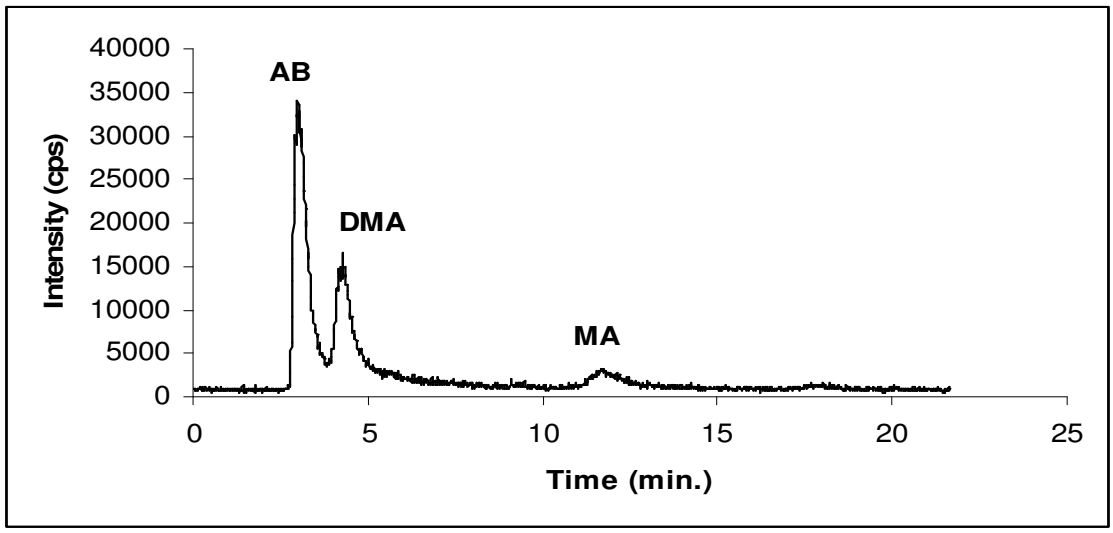

Figure A4.4.6 HPLC-ICP-MS chromatogram of a certified urine sample (CRM NIES No. 18) 5-fold diluted with the mobile phase.

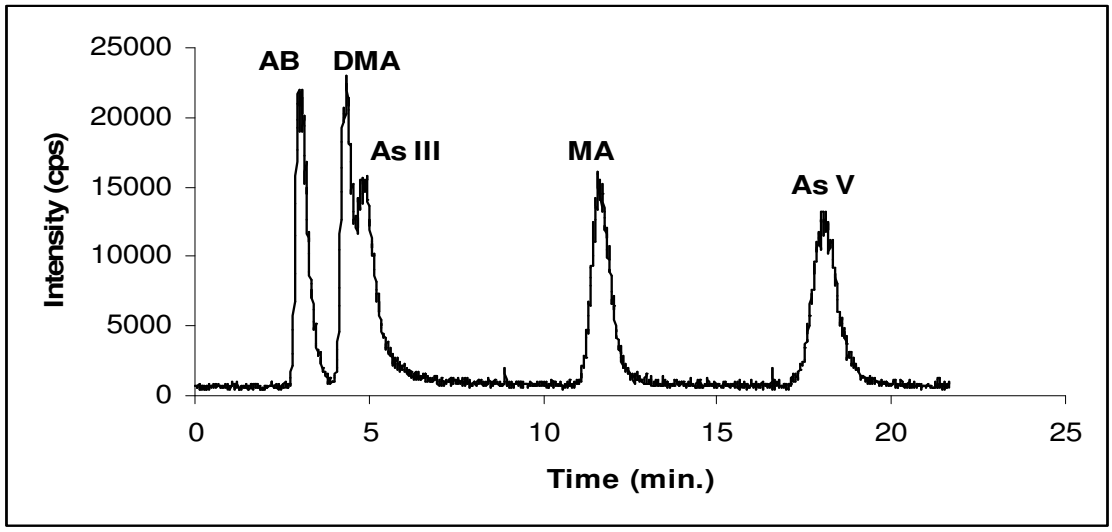

Figure A4.4.7 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppb1) of each AB, DMA, As (III), MA and As (V).

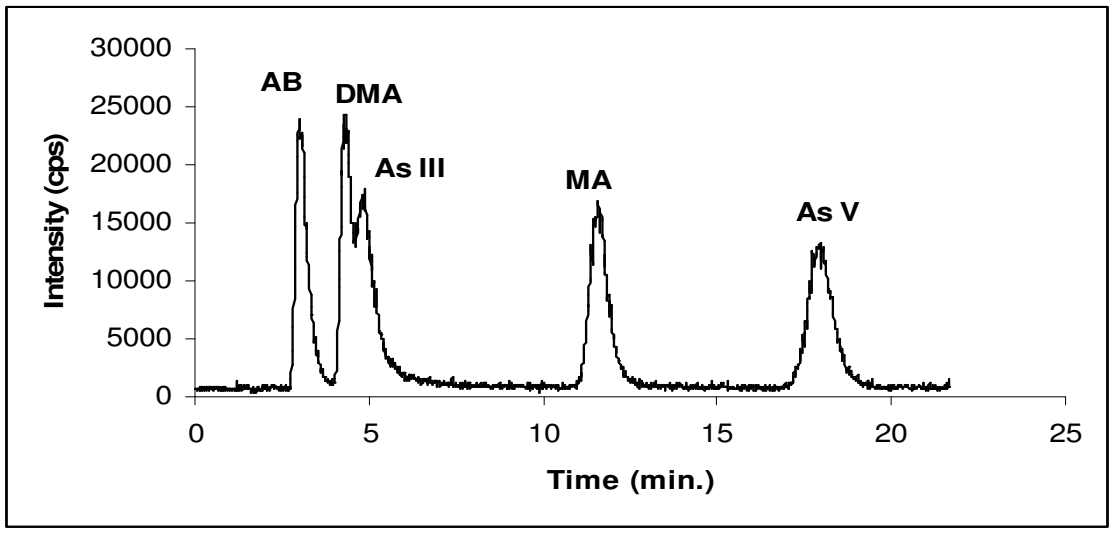

Figure A4.4.8 HPLC-ICP-MS chromatogram of a standard containing 10 µg As/l (10ppb2) of each AB, DMA, As (III), MA and As (V).

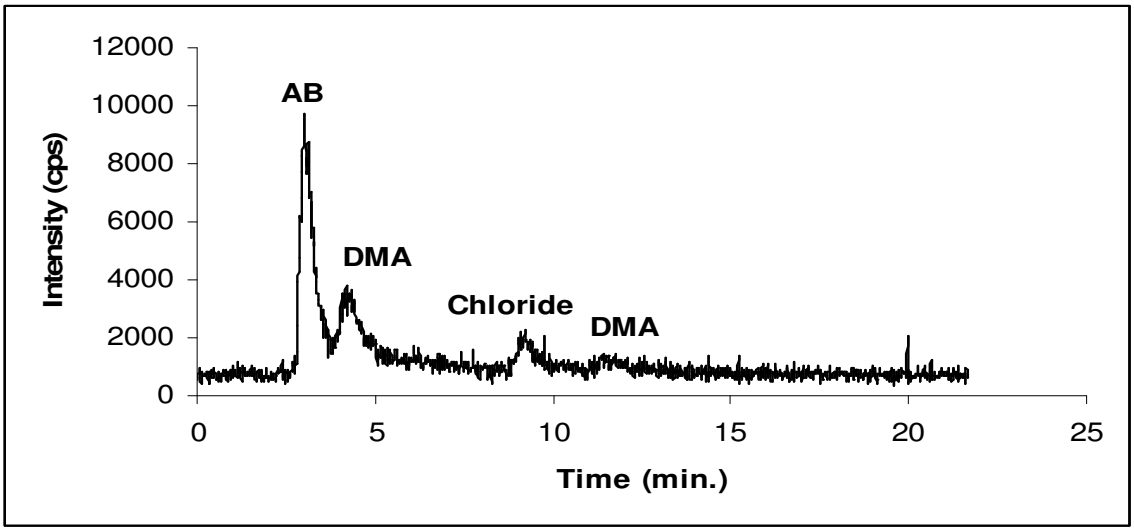

Figure A4.4.9 HPLC-ICP-MS chromatogram of a certified urine sample (UNF3), 5-fold diluted with the mobile phase.

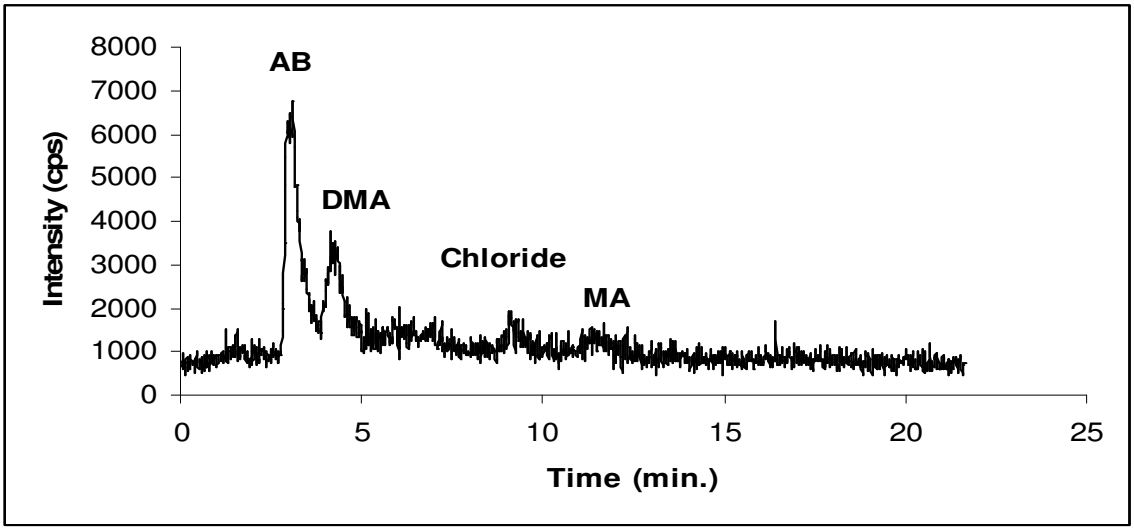

Figure A4.4.10 HPLC-ICP-MS chromatogram of a certified urine sample (UF3), 5-fold diluted with the mobile phase.

Appendix (5.1)

Dates of collection and measurements, and locations of urine samples analysis from the single volunteer monitored for a period of one year.

Sample name	Date of collection	Seafood ingestion days ago	Date of measurement (**total arsenic and selenium**)- ***at Hull University***
Au04U	23/08/2004	2	17/08/2005
Sep04U	23/09/2004	7	17/08/2005
Oct04U	28/10/2004	7	17/08/2005
Nov04U	22/11/2004	2	17/08/2005
Dec04U	20/12/2004	2	17/08/2005
Jan05U	20/01/2005	1	17/08/2005
Feb05U	22/02/2005	1	17/08/2005
Mar05U	24/03/2005	1	17/08/2005
Apr05U	22/04/2005	14	17/08/2005
May05U	20/05/2005	14	17/08/2005
Jun05U	27/06/2005	7	17/08/2005
Jul05U	27/07/2005	21	17/08/2005